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Bactericidal and virucidal ultrathin films assembled layer by layer from polycationic *N*-alkylated polyethylenimines and polyanions

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ABSTRACT

In this work, we designed contact-killing ionically cross-linked polymeric thin films using Layer-by-Layer (LbL) technology. A polycation, *N,N*-dodecyl,methyl-polyethylenimine, with microbicidal activity was layered with a polyanion, such as poly(acrylic acid), to create LbL films highly effective against both airborne and waterborne *Escherichia coli* and *Staphylococcus aureus* (Gram negative and positive bacteria, respectively), as well as influenza A/WSN (H1N1) virus. The dependence of the microbicidal activity on the pH during and post-assembly of LbL film formation, the nature of the polycation and polyanion, the number of layers in the LbL film, and other experimental variables was investigated quantitatively.

1. Introduction

Everyday items handled by people (e.g., doorknobs, handles, keyboards, and elevator buttons) are inhabited by various bacteria and viruses, some of which can spread disease. If such objects could be made microbicidal without altering their functionality and appearance, additional means of managing the spread of disease would result. Bacteria such as *Staphylococcus aureus* and *Escherichia coli* are among the most common human pathogens, and the rise of their antibiotic-resistant strains, e.g., methicillin-resistant *S. aureus* (MRSA), has become a serious problem. Furthermore, annually 5–20% of the U.S. population are infected with the influenza (flu) virus; as a result, over 200,000 people are hospitalized and about 36,000 people die each year [1]. The flu problem is even more serious when a new strain of the virus, such as the so-called swine flu (H1N1), becomes infectious to humans. Many bacterial and viral diseases can spread from person to person via contact with commonly handled objects; therefore, if their surfaces can be made bactericidal and virucidal, the extent of this spread would be reduced. In particular, eliminating live bacteria on surfaces of medical implants, thus preventing biofilm formation would be a major advancement in the biomedical field.

Existing bactericidal coatings typically incorporate microbicidal agents like silver ions [2-4], antibiotics [5], or other drugs [6], that leach into the environment. A disadvantage of this approach is that the embedded agents will eventually be exhausted, leading to limited functional lifetimes. Furthermore, leachable coatings are not desirable when the leached microbicidal agent is toxic or can lead to resistant microbes.

Recently, a new, non-releasing microbicidal strategy has been developed [7–11]. This approach utilizes hydrophobic polycations, either covalently attached as a result of a multi-step derivatization procedure or deposited (painted) onto surfaces to disrupt the bacterial membranes and inactivate influenza viruses on contact [2,12–14]. Although the hydrophobic polycations can be physically applied to surfaces from solution [10], this "painting" process cannot easily coat geometrically complex surfaces and at least micron-thick films are required for maximal bactericidal activity [15]. Also, these polymeric films can peel off or be scraped off the surface. To address these potential shortcomings, in the present study we employed the layer-by-layer (LbL) self-assembly approach [16,17].

With LbL technology, surfaces of various shapes can be coated with conformal ultra-thin films whose surface properties can be systematically controlled through film composition and morphology [17,18]. LbL technology involves sequential adsorption of multivalent species (molecules, polymers, nanoparticles, etc.) with complementary

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functional groups utilizing electrostatic or other non-covalent interactions, such as hydrogen bonding [19–21]. Owing to its ease of application and low environmental impact, LbL technology has found a broad range of applications, including biomedical materials [5,20,22], membranes and electrodes for energy applications [23,24], and electro- or magnetoresponsive surfaces [25,26].

LbL films are sensitive to assembly (pH and ionic strength) conditions; as a result, their structure and composition depend on the film building process [27]. In addition, the film structure can also be modified post-assembly by exposing the film to conditions different from those used during film assembly. For example, LbL films built from polyallylamine and poly(4-styrene sulfonate) could be made bactericidal by manipulating assembly and post-assembly pH conditions (i.e., lowered pH) [28]. Although the resultant LbL films result in high activity against Gram positive bacteria, they are not as effective against Gram negative bacteria. Although other bactericidal LbL films have been developed, most of them work by incorporating and releasing such bactericidal agents as silver ions [2–4,29], quaternary ammonium salts [2], titania [30], chitosan [31,32], antibiotics [5], and enzymes [33]. LbL films have also been designed to limit adhesion and viability of bacteria by modifying the chemical and physical properties of the surface [34].

In this work, we demonstrate that by incorporating polymeric hydrophobic quarternary ammonium salts with high bactericidal activity into LbL films, we can harness the potential of these polycations, while achieving high and broad-spectrum bactericidal activity in nanometer-scale coatings. Finally, we report the first use of LbL films to inactivate influenza virus.

2. Materials and methods

2.1. Synthesis of polymers

Poly(2-ethyl-2-oxazoline) (M_w of 500 kDa), 1-bromododecane, 1-bromohexane, 1-bromobutane, iodomethane, *tert*-amyl alcohol, and other chemicals and solvents were from Sigma–Aldrich. Linear *N,N*-dodecyl-methyl-PEI (DMLPEI) was synthesized as previously described [35]. In short, linear PEI (LPEI) (M_w of 217 kDa) was produced by deacylation of poly(2-ethyl-2-oxazoline) [36]; the resultant LPEI was dissolved in water, precipitated with aqueous KOH, filtered, and washed repeatedly with water. The resultant deprotonated LPEI was then alkylated first with 1-bromododecane (96 h at 95 °C) and then with iodomethane (24 h at 60 °C) to produce the end product DMLPEI. Syntheses of linear *N,N*-hexyl-methyl-PEI (HMLPEI) and linear *N,N*-butyl-methyl-PEI (BMLPEI) were similar, except that LPEI was alkylated with 1-bromohexane (24 h at 95 °C) and 1-bromobutane (24 h at 95 °C), respectively. As for *N,N*-dimethyl-PEI (MMLPEI), LPEI was alkylated by addition of iodomethane for 24 h at 60 °C. The structures of these polymers are depicted in Fig. 1A.

PAA (M_w of 50 kDa; Polysciences) was also used to acylate the $-NH_2$ group of dopamine (DOPA; Sigma); 15% of the carboxyl groups of PAA were functionalized with DOPA (Fig. 1B).

2.2. LbL film assembly

LbL films were assembled on rectangular 2.5 cm × 3.0 cm silicon substrates (Silicon Quest International) with a programmable Carl Zeiss HMS slide stainer. Substrates were first plasma-etched in oxygen using a Harrick PDC-32 G plasma cleaner on high RF for 1 min and then immediately immersed into a solution of a 1 mg/ml of polycation dissolved in an organic solvent for at least 10 min. Most of the polycations used in this work only dissolve in organic solvents: DMLPEI was dissolved in butanol, HMLPEI in propanol, and BMLPEI in propanol. MMLPEI was the only polycation that was soluble in water. The LbL film was built up by alternating the deposition of a polycation and a polyanion; the latter included PAA, poly(Na 4-styrene sulfonate) (SPS, M_w of 70 kDa; Sigma–Aldrich), poly(Na vinyl sulfonate) (PVS; Sigma–Aldrich), poly(methacrylic acid) (PMA, M_w of 100 kDa; Polysiences), and poly(styrene-*alt*-maleic acid) (PSMA; M_w of 350 kDa; Sigma Aldrich). The polycation solutions used for film construction were at a concentration of 1 mg/ml. Solutions of PAA, PAA-DOPA, PMA, and PSMA used were at a concentration of 2 mg/ml in 0.1 M sodium acetate buffer, pH 5.1. PAA, PMA, and PSMA solutions at pH 3.0 and pH 7.0 were pH adjusted using 1 M HCl and 1 M NaOH, respectively. SPS and PVS solutions were at 2 mg/ml in 0.1 M NaCl and in deionized water, respectively.

LbL films with the bilayer architecture of $(Polycation/Polyanion)_n$ was built, where n is the number of bilayers and polycation and polyanion could be any of those mentioned above. A bilayer would include a deposition of a layer of a polycation, followed by a layer of a polyanion; for example, a 1.5 bilayer film will have

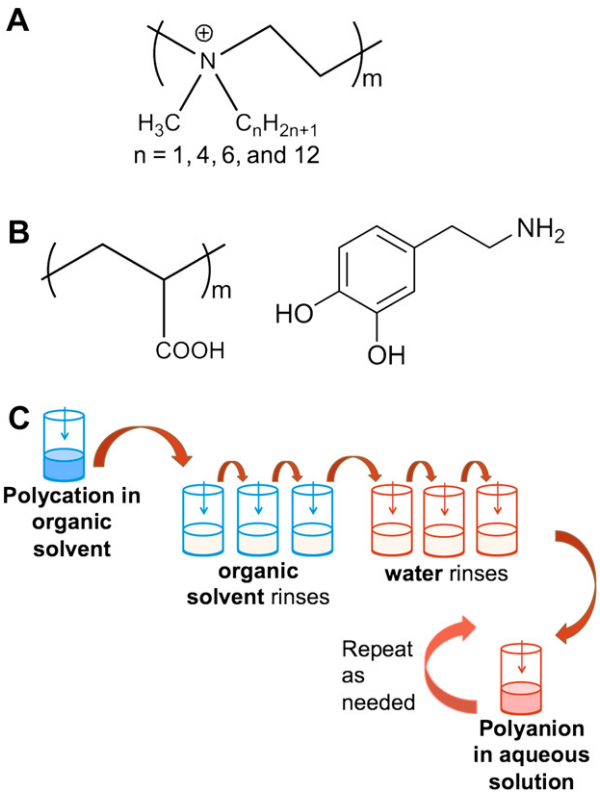

Fig. 1. (A) Structure of microbicidal polycations with various alkyl chain lengths ($n = 1$, 4, 6, and 12); (B) Structure of polyacrylic acid (PAA) and dopamine (DOPA) used for acylation between amine group of DOPA and carboxylic group of PAA; (C) Schematic of the modified LbL dipping process that alternates between an organic solvent for the polycation and an aqueous solution for the polyanion.

a complete bilayer deposited, followed by a layer of polycation on top. The following program was used to buildup a bilayer: 20 min of dipping in a polycation solution, followed by three rinses in the organic solvent used to dissolve the polycation (1 min, 30 s, and 30 s, respectively), then three rinses in deionized water (1 min, 30 s, and 30 s, respectively), followed by a 20 min dipping in a polyanion solution, then three rinses in deionized water and three rinses in organic solvent (Fig. 1C). This program was repeated until the desired number of bilayers was obtained. To be subjected to acid treatment, the built LbL films were immersed in pH 2.5 water for 3 h, rinsed vigorously in three separate rinses of deionized water, and dried gently with nitrogen gas.

2.3. LbL film characterization

Thicknesses of LbL films were measured using a spectroscopic ellipsometer (Woollam M-2000D) and verified using a surface profilometer (KLA Tencor P-16). The surface morphology and roughness of the LbL films were observed using an atomic force microscope (Nanoscope IIIa; Digital Instruments) in tapping mode and a scanning electron microscope (JEOL 6320-HR).

Fourier transformed infrared (FT-IR) spectra of $(DMLPEI/PAA)_{50}$ films with PAA at pH 3.0, 5.0, and 7.0 were acquired using a Nexus 6700 FT-IR (Thermo-Nicolet). Films with such large number of bilayers (50) were used to acquire the data because films typically investigated in this work (less than 4.5 bilayers) did not have sufficient material for the spectrophotometer to detect.

2.4. Airborne bacterial assay

The bacterial strains used herein were *S. aureus* (ATCC, 25923) and *E. coli* (*E.coli* genetic stock center, CGSC4401). Bactericidal activities of the LbL films were tested based on a previously developed protocol [37]. Briefly, *S. aureus* was grown overnight in cation-adjusted Mueller Hinton Broth II (CMHB) (Difco, BD) and diluted to 5×10^6 cells/ml. The diluted bacterial suspension was sprayed onto samples (~10 ml/min) using a gas chromatography sprayer (VWR International, cat. No. 21428-350); samples were incubated at room temperature for 2 min, placed in a Petri dish, and covered with a slab of solid growth agar made from CMHB media and BactoAgar (Difco, BD). The Petri dishes were incubated overnight at 37 °C and bacterial colonies on the surface of the samples were counted by hand if there were

a few colonies; alternatively, ten digital images per sample were taken with a 4× objective using a microscope (Axioskop 2 MAT microscope, Carl Zeiss), and the total number of colonies was extrapolated to the whole area of the sample. The same procedure was used for *E. coli* except that it was grown overnight in LB-Miller broth (VWR), diluted to 5×10^7 cells/ml, and the agar plates used to incubate the samples were made of 1% LB agar (VWR). Bactericidal activity was calculated by comparing the number of colonies on negative control slides and on the samples. Negative controls used were cleaned silicon substrates. Positive controls were silicon substrates painted with a solution of 50 mg/ml DMLPEI dissolved in butanol as described previously [35].

2.5. Waterborne bacterial assay

S.. aureus was grown overnight at 37 °C in cation-adjusted Mueller Hinton Broth II (CMHB) (Difco, BD); the culture was then centrifuged at 2700 rpm for 10 min, washed, resuspended in deionized water, and diluted to 10^6 cells/ml. LbL films coated substrates were then incubated with the resultant solution at room temperature for 2 h to promote bacteria adhesion onto the surface. Samples were rinsed thrice in deionized water and incubated under a solid slab of agar (Difco BD) overnight at 37 °C. Bare Si substrate was used as the negative control. The same procedure as in the airborne assay was used for counting the bacterial colonies on the samples and controls. The results were presented as a colony density, which is defined as average number of colonies on sample/average number of colonies on silicon control times 100%; therefore, the colony density of a Si substrate control was always equal to 100% [7].

2.6. Determination of virucidal activity

The influenza virus strain used was A/WSN/33 (H1N1); the virus was grown in the Madin–Darby Canine Kidney (MDCK) medium from ATCC [35]. The protocol to test the samples for virucidal activity was that described previously [37]. Briefly, a sample was placed in a Petri dish and a 10 μl droplet of a virus solution ($\sim 1.6 \times 10^5$ pfu/ml) was placed in the center of the sample; a sandwiched system to spread the virus droplet was formed by putting a plain silicon substrate on top of the sample. This system was incubated at room temperature for 30 min; then the top substrate was lifted, and the virus droplet exposed sides were washed thoroughly with PBS. Lastly, a plaque assay was performed to determine whether the samples were effective in inactivating the virus. The virucidal activity was determined by comparing the virus titers obtained from plaque assay of the negative control substrate and of the sample (these control samples were the same as described above for determining bactericidal activities).

2.7. Adhesion and non-leaching test of LbL films

The mechanical integrity of the film on surfaces was tested using a 3M Scotch tape which was attached to the film and then removed in one motion. The test was performed on $(DMLPEI/PAA3.0)_{4.5}$ films. The thickness and bactericidal activities of the films before and after the test were measured.

A modified Kirby–Bauer assay described previously was used to test samples for non-leachability [5]: a sample coated side down was placed on *S. aureus* streaked agar plate and incubated overnight at 37 °C.

2.8. In-vitro cytotoxicity assay

Murine pre-osteoblast cell line MC3T3 (ATCC) was seeded on coated (with $(DMLPEI/PAA3.0)_{1.5}$ or $(DMLPEI/PAA3.0)_{2.5}$) and non-coated glass substrates (control), and these substrates were then placed in wells of a 6-well plate. 100,000 cells/well was incubated for 24 h and then labeled with live/dead fluorescent stains (Live/Dead Viability/Cytotoxicity Kit for mammalian cells; Invitrogen). A series of 10 digital images were taken of cells at 10× magnification using a fluorescence microscope (Axioskop 2 MAT microscope, Carl Zeiss). The numbers of live and dead cells were counted on each sample, and the percentage of cell viability was computed relative to control.

3. Results and discussion

3.1. Airborne bactericidal activity of LbL films

The polycations used to build the LbL films in this study (Fig. 1A) varied in hydrophobicity with the length of their *N*-alkyl chain; in contrast to the polyanions used (Fig. 1B), these polycations were insoluble in water and thus were dissolved in butanol or propanol. The microbicidal activity of the LbL films was examined as a function of the polyanion and the pH of the polyanion aqueous solution during assembly. The polyanion charge density and whether its monomer was a weak or a strong acid, was anticipated to be important because the interactions between the polyelectrolytes govern the availability and density of the positive charges and the conformation of the hydrophobic groups on the surface.

Because LbL assembly typically involves alternation between aqueous solutions, a modified version of the LbL protocol that involved dipping from an organic polycation solution, followed by solvent and then aqueous rinses was introduced, as described in Section 2. Fig. 2 depicts the growth curves and roughness of $(DMLPEI/PAA)_n$ films built with PAA solutions at pH 3.0 and 5.0. The films exhibit an initial lag growth phase (thickness not significantly increasing) studied extensively in the LbL literature [38], followed by a linear growth beyond 4.5 bilayers. The roughness data for the $(DMLPEI/PAA)_n$ films (Fig. 2) show that for films up to 4.5 bilayers, the thickness and roughness values were statistically the same (from 4 to 10 nm), supporting the initial patchy growth period reported by others [39]. As the number of bilayers was increased further, however, the roughness of the film grew. We observed that the bactericidal activity of $(DMLPEI/PAA)_n$ films rose with increasing number of bilayers; 100% bactericidal activity was achieved at 14.5 bilayers of $(DMLPEI/PAA)_n$ built with PAA at pH 5.0.

We then investigated the dependence of bactericidal activity on the pH of the PAA solution used during film assembly. As seen

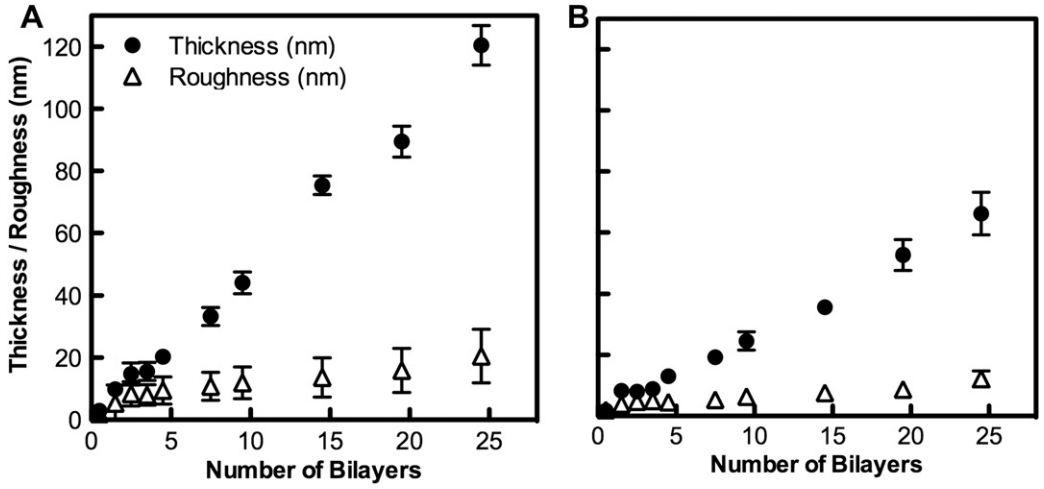

Fig. 2. Growth curve and roughness of $(DMLPEI/PAA)_n$ prepared with (A) PAA at pH 3.0, and (B) PAA at pH 5. 0 showing initial patchy growth of the films, and linear growth beyond 4.5 bilayers.

in Fig. 3A, by adjusting the pH of the PAA solution used for film deposition the level of bactericidal activity of the films can be changed significantly, achieving complete bactericidal activity with only 1.5 bilayers of deposition for $(DMLPEI/PAA)_n$ with PAA at pH 3.0. We can thus achieve the same level of activity as the microbicidal "painted" films (micron thick) with a much thinner (~10 nm) LbL film using the same microbicidal polycation, DMLPEI. Because the degree of ionization of weak polyacids is highly pH-dependent, the conformation of the PAA chains should also change from a relatively flat and thin random coil at high pH to chain arrangements that take on loops, coils, and brushes forming thicker layers when less charged at low pH [40,41]. Fig. 2 shows that LbL films built with PAA at pH 3.0 are thicker than those built at pH 5.0.

When only one layer of the DMLPEI (0.5 bilayer of the film) was deposited on a negatively charged Si wafer, no bactericidal activity was observed. This observation is likely because the negatively charged plasma-treated surface of the Si wafer induced deposition of the DMLPEI layer as a surface-conformal coating, with many of the positive charges tightly associated with the surface negative charges and thus little brush layer generated at the surface. When a PAA at pH 3.0 layer was then deposited, followed by another layer of DMLPEI thus producing a $(DMLPEI/PAA)_{1.5}$ film, the bactericidal activity of the film jumped dramatically to 100% (Fig. 3A). This observation is in agreement with results reported in literature finding that the conformation of the previously adsorbed layer greatly affects the conformation of the next polymer layer [27,40]. In this case, having a weakly charged PAA layer yields the subsequent DMLPEI layer with a thicker and loopier brush-like architecture with many of its positive charges available to interact with bacterial cell membranes. Note that only the pH of the polyanion solution could be changed because the polycation used was dissolved in an organic solvent.

The finding that the bactericidal activity of our LbL films against *S. aureus*, increases as the pH of PAA solution used for assembly is lowered (Fig. 3A) is consistent with the current view that mobile positive charges plus hydrophobic alkyl chains of a certain length are necessary for bactericidal activity [2,13,28,42,43]. It is believed that there are initial electrostatic interactions between the cationic surface and the negatively charged bacterial cell membranes; subsequently, the hydrophobic alkyl chains can diffuse through the lipid bilayer, thus undermining the integrity of the membrane and eventually leading to the death of the bacteria. Previous research has also shown that multiple positive charges are required to permeate cell membranes [44]; the arrangement, accessibility, and mobility of these charged groups on the surface are important as well [45]. A schematic illustrating the likely conformations of the PAA chains and the microbicidal polycation chains is depicted in Fig. 4A. A transition is observed from thicker and more brush-like PAA layers at low pH that leave more available positive charges on the surface for interaction with the bacterial cell membrane to flat, highly charged PAA layers at higher pH that undergo greater electrostatic interactions with DMLPEI. FT-IR spectra shown in Fig. 4B support this proposed trend. LbL films made from PAA at pH 3.0, 5.0, and 7.0, showed decreasing intensity of the COOH acid band (C=O stretching of COOH, $\nu = 1710–1700\ cm^{-1}$) and concomitantly increasing intensity of the COO^- band (asymmetric stretching band of COO^-, $\nu = 1565–1542\ cm^{-1}$) as the pH of PAA is increased (Fig. 4B).

Our films were found effective against both the Gram positive *S. aureus* and the Gram negative *E. coli* bacteria (Fig. 3B). The difference in the composition of the cell walls of these two types of bacteria should influence the way that positive charges coupled with hydrophobic alkyl chains interact with them [46]. Gram positive bacteria have a simple cell wall consisting of a thick

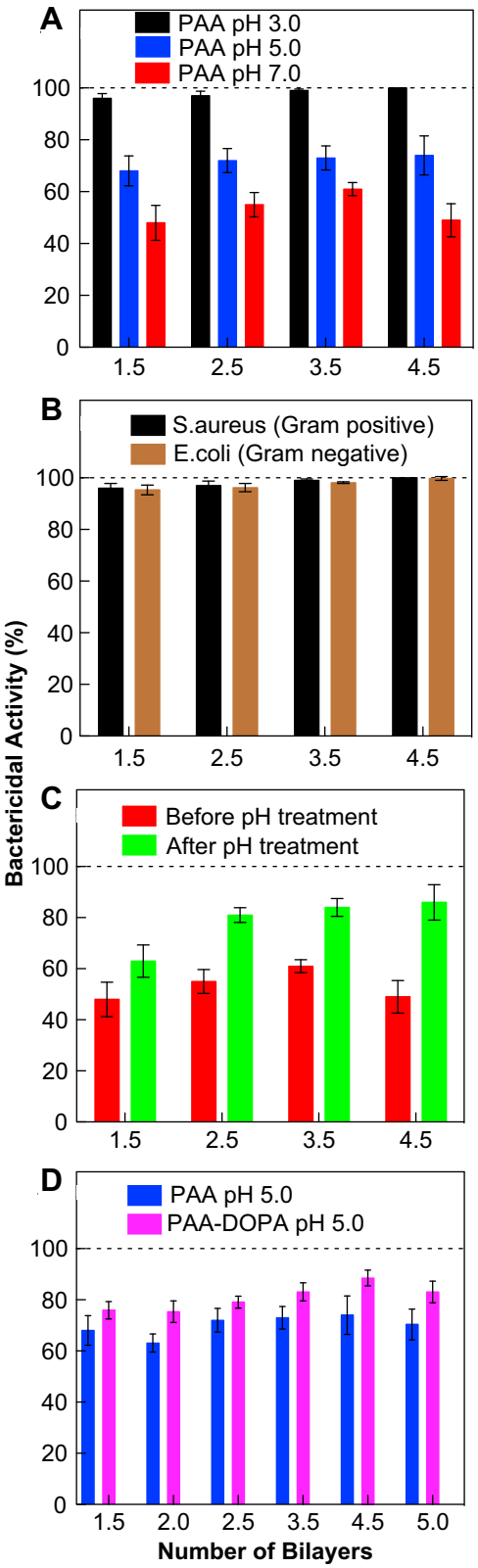

Fig. 3. Activities of $(DMLPEI/PAA)_n$ LbL films against airborne bacteria. (A)1.5–4.5 bilayers films built with PAA at pH 3.0, 5.0, or 7.0; (B) Activities of 1.5–4.5 bilayers $(DMLPEI/PAA)_n$ films against *S. aureus* and *E. coli*. (C) Activity of $(DMLPEI/PAA)_n$ films prepared at pH 7.0 before and after pH treatment; (D) Comparison of activity of 1.5–5.0 bilayers $(DMLPEI/PAA)_n$ (prepared at pH 5.0) and $(DMLPEI/PAA\text{-}DOPA)_n$ films (also at pH 5.0); (D) also shows the comparison of activity of a 1.5 and 2.0 bilayers film and a 4.5 and 5.0 bilayers film illustrating the activities of films with either DMLPEI or PAA as the topmost layer. All films were tested with *S. aureus*, except as noted in (B) where *E. coli* was used as well.

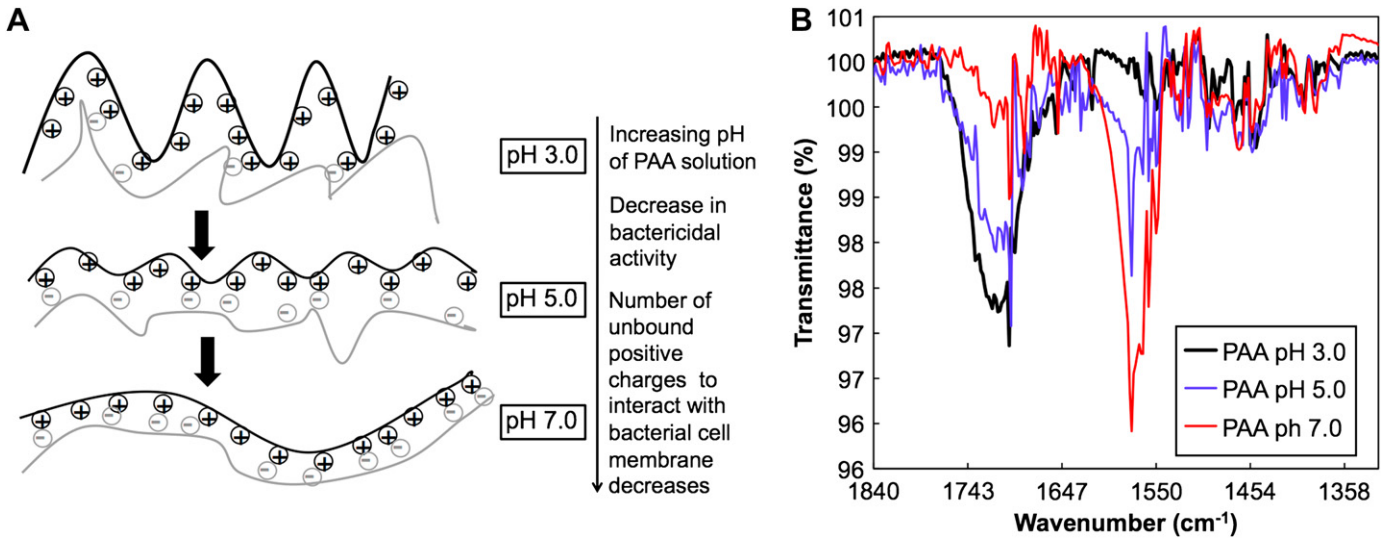

Fig. 4. (A) Schematic representation of the change in conformation of the polymer chains as the pH of the PAA solution used during film assembly is increased. At pH 3.0, the PAA chains are mostly uncharged resulting in a more loopy chain conformation of the polycation DMLPEI, with most of its positive charges available to interact with bacterial cell membranes. As the pH of the PAA solution is increased, the PAA chains become more negatively charged, resulting in more ionic cross-linking with the DMLPEI chains, thus decreasing their number of available positive charges for interaction with bacterial cell membrane; (B) FT-IR spectra of $(DMLPEI/PAA)_{50}$ films with PAA at pH 3.0, 5.0, and 7.0 during film assembly. Two distinct bands of the carboxylic acid group of PAA were observed: asymmetric stretching band of COO^- ($\nu = 1565–1542\ cm^{-1}$), and C=O stretching of COOH ($\nu = 1710–1700\ cm^{-1}$).

peptidoglycan layer, while Gram negative bacteria have cell walls made out of a lipopolysaccharide layer, a thin peptidoglycan layer, and the periplasmic space [47]. This extra lipopolysaccharide layer is apparently capable of protecting Gram negative bacteria, making it harder to kill them using just positive charges [48]. In addition, Gram negative bacteria have been shown to change their outer membrane composition to provide extra protection when in contact with quaternary ammonium compounds [49]. Therefore, to exert a broad spectrum bactericidal activity, both positive charges and hydrophobic alkyl chains, as exist in our LbL films, appear necessary.

To further test the view that having mobile positive charges is important for enhanced bactericidal activity, we subjected $(DMLPEI/PAA)_n$ films built from PAA solution at pH 7, to water at pH 2.5, as previously investigated by Lichter and Rubner, who subjected $(SPS/PAH)_n$ films to a low pH, and saw an increase in bactericidal activity of their films [28]. Fig. 3C shows that the bactericidal activity of our films increases after exposure of the films to low pH; when the films are exposed to a low pH, some of the carboxylate (COO^-) groups on the PAA chains are protonated to carboxylic (COOH) groups, resulting in conformational changes to the PAA chains, and consequently, the DMLPEI chains [27]. Ultimately, the polymer chains become more mobile with the lowered charge of the PAA chains, leading to less ionic crosslinking with the positive charges on the DMLPEI chains. Thus not only surface coverage (increasing with the number of bilayers) but also the number of mobile positive charges is important for the bactericidal activity of the LbL films. Indeed SEM and AFM images of the films taken before and after the pH treatment (Fig. 5) reveal that the surface coverage increases, suggesting rearrangements of looser polycationic chains. After the pH treatment, the roughness of the film decreased from 4.72 ± 1.20 nm to 2.08 ± 0.87 nm. These data indicate that treating $(DMLPEI/PAA)_n$ films built originally with PAA at pH 7.0 post-assembly with a pH 2.5 aqueous solution raised the bactericidal activity of the films, although not to the level of the films originally built at a low pH (namely, PAA solution at pH 3.0); thus the ionic cross-linking within the LbL films is only partially reversible.

By modifying PAA with dopamine (DOPA), the number of carboxylate (COO^-) groups available to form ionic bonds with DMLPEI's positive charges is effectively reduced and the bactericidal activity is increased (Fig. 3D). Functionalizing PAA with DOPA, which reduces the charge density of the PAA chains, presumably results in a loopier PAA chain conformation, as in the case of films built with PAA at low pH assembly conditions, thereby influencing the conformation of the next adsorbed DMLPEI layer and yielding a brush-like layer.

There are also indications of interpenetration between the polyelectrolyte layers in the LbL films discussed here. For example, for the $(DMLPEI/PAA)_2$ and $(DMLPEI/PAA)_5$ films, it did not matter whether PAA or DMLPEI was the topmost layer (Fig. 3D). Instead of forming well-stratified layers, those formed within the LbL films interdigitate, as is typical for polyelectrolyte multilayers [50].

We also found that the bactericidal activity of the LbL films was influenced by the hydrophobicity of the polycation, i.e., the length of its alkyl chains. Using PAA solutions at pH 3.0 and pH 7.0 and polycations with various alkyl chain lengths (Fig. 6A), we found that the bactericidal activity against *S. aureus* varied significantly on the alkyl chain length. For example, films built with linear *N,N*-dimethyl-PEI (MMLPEI, $n = 1$) and *N,N*-butyl,methyl-PEI (BMLPEI, $n = 4$) were significantly less bactericidal than those built with *N,N*-hexyl,methyl PEI (HMLPEI, $n = 6$) and DMLPEI. To examine the activity against Gram negative bacteria, for which the size of the alkyl chain is thought to be critical [28], films made with BMLPEI and MMLPEI as polycations, and PAA at pH 3.0 and pH 7.0 as polyanions were tested against *E. coli*. The bactericidal activity against *E. coli* was found to be lower compared to that against *S. aureus* for $n < 6$ (Fig. 6B). In addition, films built using either linear or branched non-alkylated PEI as a polycation and PAA (at pH 3.0) were impotent against both bacteria. Therefore, hydrophobicity, along with a high positive charge density, is necessary for bactericidal activity.

Next we examined the dependence of bactericidal activity of the LbL film built with DMLPEI as the polycation on the strength of the acid in the polyanion used. Films built using strong polyacids as poly(4-styrenesulfonate) (SPS) and poly(vinylsulfonate) (PVS) displayed no bactericidal activity. Because strong polyacids are highly

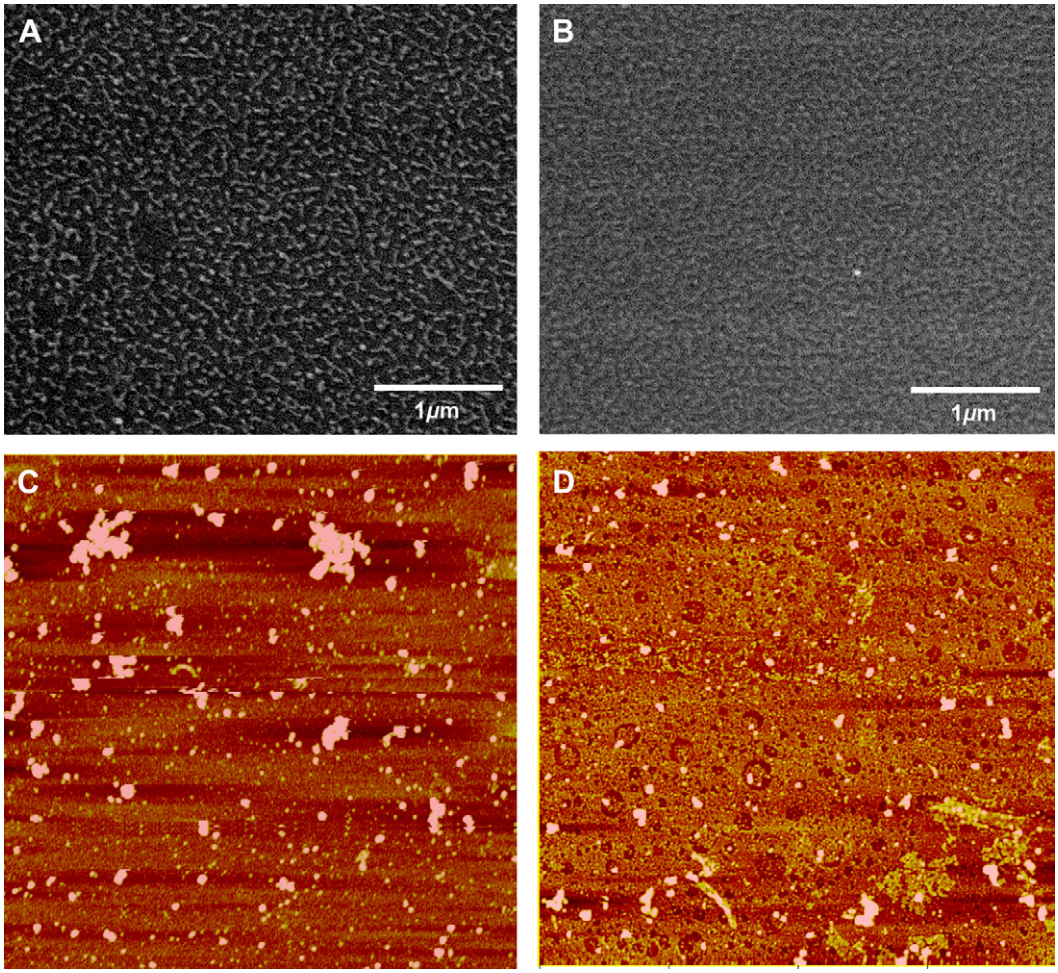

Fig. 5. SEM images of $(DMLPEI/PAA)_{2.5}$ films prepared at pH 7.0 (A) before and (B) after pH treatment, showing the increase in coverage of the film post pH treatment. AFM images (10 μm × 10 μm scan) of $(DMLPEI/PAA)_{4.5}$ films prepared at pH 7.0 (C) before and (D) after pH treatment, also showing more uniform coverage of the film after low pH treatment; *z*-scale = 25 nm.

ionized, most of the positive charges on the DMLPEI chains should be tightly bound to their negative charges resulting in few mobile positive charges available to disrupt bacterial cell membrane. This effect persisted even when salt was added to the sulfonated polyion solution to reduce the polyanion-polycation electrostatic attraction via ionic screening.

Another interesting observation was that the hydrophobicity of the weak polyacid used for film assembly affected the bactericidal activity as well (Fig. 6C). While LbL films built with a slightly more hydrophobic poly(methacrylic acid) (PMA) had similar bactericidal activity as those built using PAA, with a much more hydrophobic poly(styrene-*alt*-maleate) (PSMA) the bactericidal activity dropped significantly. The bulky styrene groups of this strong polyacid could change the polyanion-polycation interaction changing the presentation of the polycation on the surface and also interact with the DMLPEI's alkyl chains preventing them from functioning as "brushes" on the surface, thus decreasing the activity of the films.

3.2. Activity of LbL films against waterborne bacteria

Next we explored the possibility of using our microbicidal LbL films as permanent coatings for biomedical implants to prevent bacterial attachment in aqueous environments. To this end, 1.5 bilayer and 2.5 bilayer $(DMLPEI/PAA)_n$ films built at pH 3.0, 5.0, and 7.0 were tested against waterborne *S. aureus* and found to be effective in preventing bacterial attachment onto the surface relative to a bare silicon substrate control. As seen in Fig. 7, the films made at pH 3.0 were most effective in preventing bacteria from attaching to the surface, which is in agreement with our aforementioned airborne results.

3.3. Virucidal activity of LbL films

The most bactericidal LbL films were tested against Influenza A/WSN/33 (H1N1) virus. This virus has an outer lipid envelope [51] which may be vulnerable to the high density of positive charges and hydrophobicity presented on the LbL surfaces (as was the case with surfaces "microbicidally painted" with DMLPEI [35]).

A 1.5 bilayer $(DMLPEI/PAA)_n$ film built with PAA at pH 3.0 was found to be 60% virucidal (Fig. 8), although it was 100% bactericidal. However, the virucidal activity of the LbL films increased as we increased the number of bilayers, and reached 100% beyond 7.5 bilayers. Since the size of a viral particle (~100 nm) is at least 10 times smaller than that of a bacterium (~1 μm), the incomplete virucidal activity seen with our films at a lower number of bilayers may be due to the voids on the surface that are large enough to fit a virus but too small for a bacterium (Fig. 9).

SEM images of the initial layers of our films show that as the number of bilayers increases, the surface coverage of the films increases (Fig. 9A). At a lower number of bilayers, there are areas on

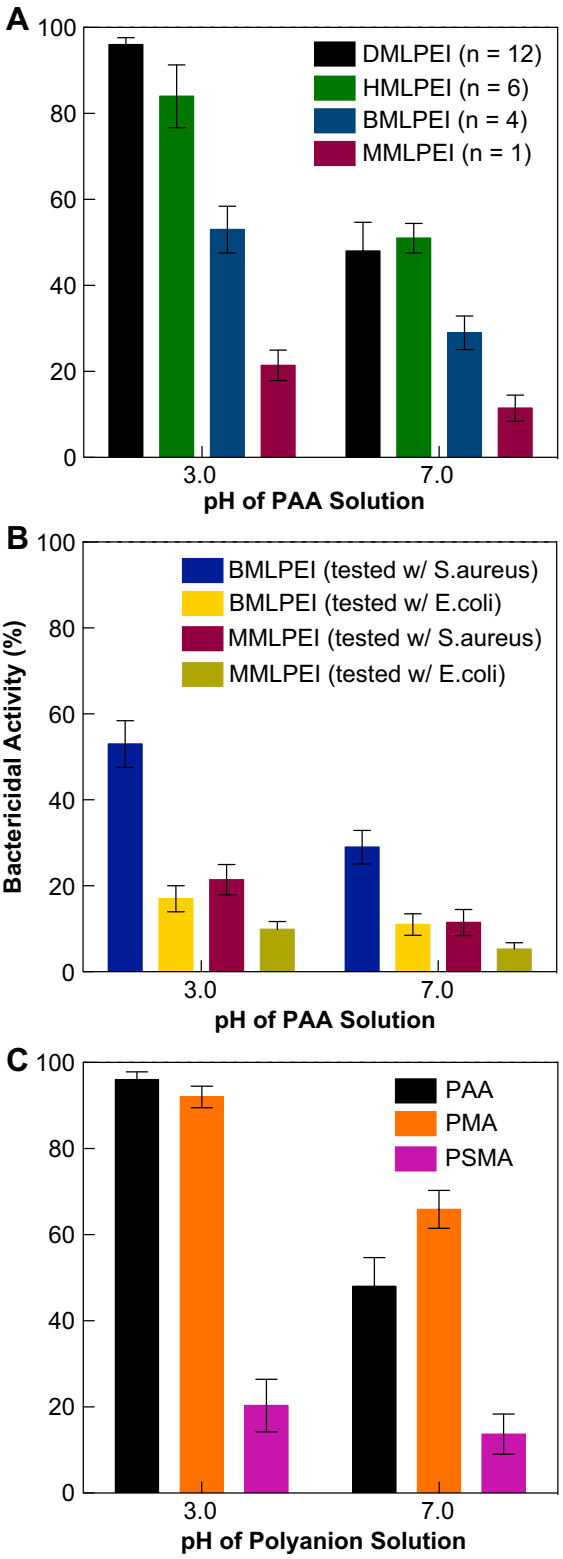

Fig. 6. All films were 1.5 bilayers. (A) Films were built with polycations of various alkyl chain lengths ranging from methyl ($n = 1$) to dodecyl ($n = 12$), with PAA as the polyanion (pH 3.0 and 7.0). (B) MMLPEI ($n = 1$) or BMLPEI ($n = 4$) films built with PAA at either pH 3.0 or pH 7.0 were tested against *S. aureus* and *E. coli*. (C) Films were built with DMLPEI and three polyanions of varying hydrophobicity.

the surface not covered by the film; this patchiness can enable a virus particle (~100 nm) to land on bare surface regions and thus not be inactivated. This bare spot is too small to fit a bacterium

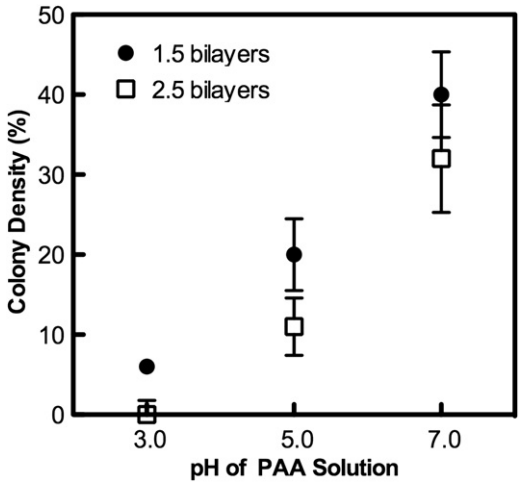

Fig. 7. Activity of LbL films against waterborne *S. aureus* comparing colony density on a bare silicon control with that on either a 1.5 or a 2.5 bilayer $(DMLPEI/PAA)_n$ films; PAA was at pH 3.0, 5.0, or 7.0; colony density on control slide is 100%.

(~1 μm), thus 100% bactericidal activity is achieved even for films with low numbers of bilayers.

3.4. Cytotoxicity, non-leaching, and adhesion tests of LbL films

To test the safety of our microbicidal LbL films, we observed mammalian cell viability when seeded on coated surfaces. To this end, an *in-vitro* cytotoxicity assay with Murine pre-osteoblast cells (MC3T3) was performed with $(DMLPEI/PAA)_{1.5}$ and $(DMLPEI/PAA)_{2.5}$ films formed at pH 3.0 using an uncoated substrate as control. The cell viability on our films was found to be indistinguishable from that on the uncoated surface, indicating that there is no apparent cytotoxicity associated with our films from this study.

The proposed mechanism for microbicidal action of DMLPEI is via a direct disruption of the microbes' membrane by hydrophobic polycationic chains upon contact with the film [9]. To establish whether our LbL films also kill on contact, rather than by leaching the polycation, a modified Kirby-Bauer assay was performed [5]. No zone of inhibition was detected beyond the film boundaries (Supplementary Figure 1) and only bacteria directly in contact with the film were killed.

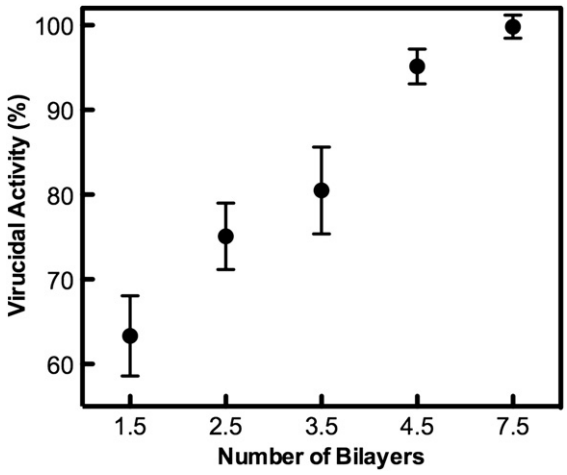

Fig. 8. Virucidal activity of the $(DMLPEI/PAA)_n$ LbL films prepared from PAA at pH 3.0 against influenza A/WSN/H1N1 virus.

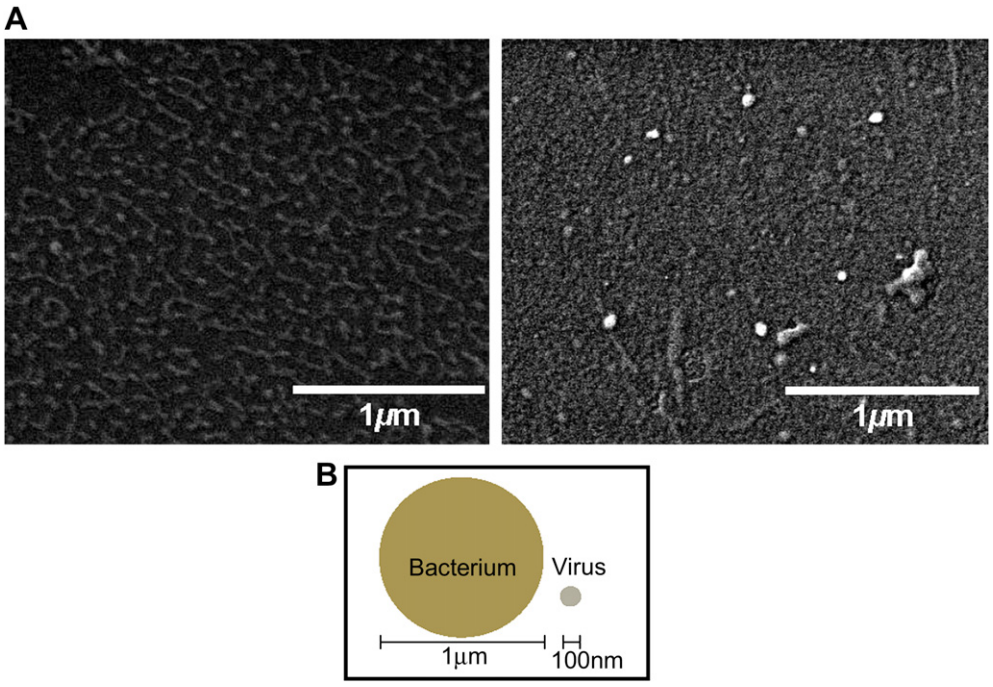

Fig. 9. (A) SEM images of 2.5 and 7.5 bilayers $(DMLPEI/PAA)_n$ films prepared from PAA at pH 3.0, showing the increase in surface coverage of films with increasing number of bilayers. (B) Relative size of a bacterium to a virus particle.

Finally, the mechanical integrity of our LbL films was tested by attaching a strip of 3 M Scotch tape onto the surface of the film and removing it quickly in a single motion. The films before and after this experiment were tested for bactericidal activity against *S. aureus*. We observed no change in the activity of the $(DMLPEI/PAA)_{4.5}$ films, built with PAA at pH 3.0, demonstrating their mechanical robustness. We also observed no difference in the measured film thickness before and after the adhesion test ($20.3\ nm \pm 1.5\ nm$).

4. Conclusions

We demonstrated herein that microbicidal functionality could be imparted onto surfaces using LbL technology by using the right combination of polycations and polyanions, as well as assembly and/or post-assembly conditions. We found that LbL films that are as thin as 10 nm were lethal to both airborne and waterborne Gram positive and Gram negative bacteria, as well as to a strain of influenza virus. Our films were effective in preventing bacterial attachment to surfaces, and thus indicate promising properties for prevention of biofilms on surfaces. While highly microbicidal, the LbL coatings were also found to be non-cytotoxic to a mammalian cell line based on cell viability assay. Coupled with their mechanical robustness, these properties bode well for practical potential of our LbL films where microbicidal and virucidal functionalities are required.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.01.119.

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