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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

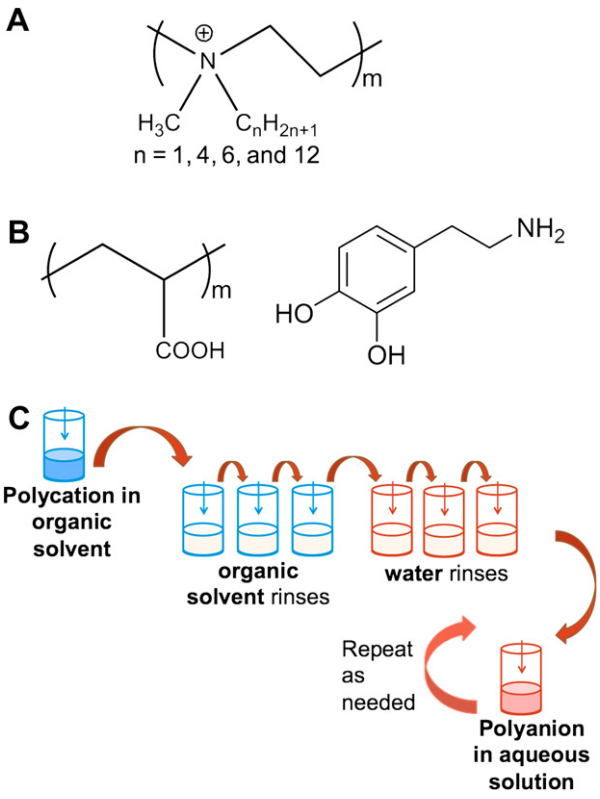

Fig. 1. (A) Structure of microbicidal polycations with various alkyl chain lengths ($n = 1$, 4, 6, and 12); (B) Structure of polyacrylic acid (PAA) and dopamine (DOPA) used for acylation between amine group of DOPA and carboxylic group of PAA; (C) Schematic of the modified LbL dipping process that alternates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