A Substrate-Independent Approach for Bactericidal Surfaces

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ABSTRACT Existing methods for imparting antibacterial performance to solid surfaces tend to either be substrate-specific or rely upon leaching modes of action that cause ecological damage. An alternative approach is outlined comprising plasmachemical functionalization of solid surfaces with poly(4-vinyl pyridine) moieties and their subsequent activation (quaternization) with bromobutane to yield bactericidal activity. These bioactive surfaces can be applied to a host of different substrate materials and are easily regenerated by rinsing in water.

KEYWORDS: Plasma polymer • thin film • antibacterial • functional nanocoating • biocidal • bioactive

1. INTRODUCTION

Antimicrobial surfaces are important for making our homes (1), hospitals (2), restaurants (3), and laboratories (4) safer places. They are required for stopping the spread of bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, Escherichia coli (*E. coli*), Salmonella, nosocomial, and community-acquired pathogens (e.g., methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE)). For instance, *Staphylococcus aureus* is frequently implicated in wound infections, osteomyelitis, endocarditis and sepsis (5). Few current commercial products exhibit suitable efficiency, endurance, and applicability to deal with such a diverse microbial onslaught. Therefore, there exists a demand for bactericidal functional coatings that are viable on a wide range of substrates and easily amenable to recharging (e.g., during a laundry wash in the case of textiles).

Biocides such as silver (6, 7), benzalkonium chloride (8), iodine (9), Irgoson (10−12), chlorhexidine (13, 14), and antibiotics including dicloxacillin (15), teicoplonin (16), minocycline with rifampin (17) and mupirocin (18) are widely impregnated into substrate surfaces and their modus operandi is gradual leaching into the surrounding medium (19). Such slow-release bioactive materials offer protection only from specific forms of bacteria. Additionally, many of the aforementioned biocides are susceptible to limited life-span, costly impregnation methods, and extensive environmental concerns stemming from biofouling and biocorrosion.

The application of diaphanous polymer layers containing active antimicrobial centers onto solid surfaces is a potential solution to these issues, since this method offers a highly cost-effective means for imparting substrate antibacterial performance without affecting the underlying bulk material properties. For instance, it has previously been reported that pyridinium-type quaternary ammonium salt resins are popular candidates for use as disinfectants because of their broad spectrum of antibacterial activity, high kill rate, and their nontoxicity toward mammalian cells (20−22). The bactericidal mechanism of these polycationic chains has been attributed to their penetration into the bacterial membrane, resulting in cell damage and death (23−25). Unfortunately, these polymers suffer from shortcomings such as bad adhesion, poor mechanical properties, and high solubility (leaching) in water. Therefore, there exists a need to securely attach these bioactive moieties onto solid substrates.

Earlier attempts aimed at addressing this challenge have included electron beam irradiation (26) and surface activated grafting (24, 27−33) of an appropriate monomer (e.g., 4-vinyl pyridine) followed by quaternization using a halocarbon to yield antibacterial activity. However, drawbacks still remain, such as substrate specificity and low grafting densities. These limitations can be overcome by utilizing pulsed plasmachemical deposition. This constitutes the generation of active sites (predominantly radicals) both at the surface and in the electrical discharge during the pulse duty cycle on-period, followed by conventional chain growth polymerization reaction pathways proceeding within each plasma extinction off-period (34). Typical time scales are on the order of microseconds and milliseconds, respectively. The level of surface functionalization can be tailored by simply preprogramming the pulsed plasma duty cycle. Functional films containing high levels of thiol (35), amine (36), pyridine (37), cyano (38), anhydride (34), hydroxyl (39), epoxide (40), carboxylic acid (41), furfuryl (42), halide (43), perfluoroalkyl (44), perfluoromethylene (45), or trifluoromethyl (46) groups have been successfully prepared in the past using this methodology. In this article, it is shown that pulsed plasmachemical deposition can be employed to impart antibacterial properties to a solid substrate while maintaining low solubility in aqueous media, Scheme 1. This is achieved in two stages comprising preferential polymerization of the vinyl carbon−carbon double bond contained in the 4-vinyl pyridine monomer during pulsed plasma deposition (retention of the pyridine ring aromatic function-

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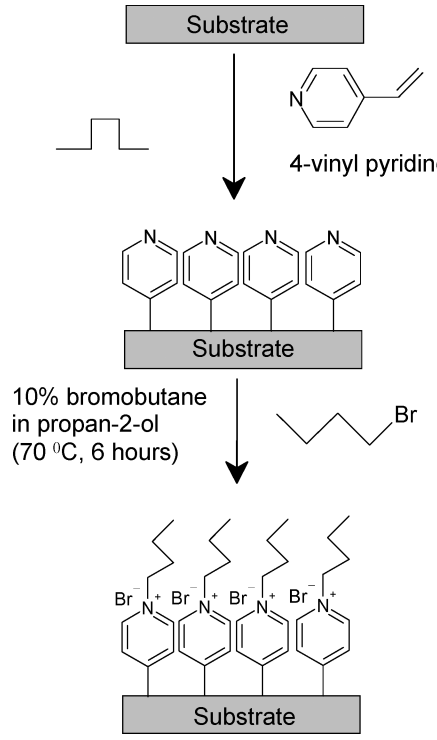
Scheme 1. Pulsed Plasmachemical Deposition of Pyridine Functionalized Solid Surfaces and Subsequent Quaternization with Bromoalkane Solution



ality), followed by quaternization of the immobilized pyridine centers with haloalkanes to yield the desired bactericidal properties.

2. EXPERIMENTAL SECTION

Pulsed plasma polymerization of 4-vinyl pyridine precursor (Aldrich, +97%, purified by several freeze−pump−thaw cycles) was carried out in a cylindrical glass chamber pumped by a rotary pump connected to a liquid nitrogen cold trap (base pressure = 1×10^{-3} mbar and leak rate = 9.9×10^{-9} mols^{-1}) (47). A copper coil wrapped around the reactor was attached to a 13.56 MHz radio frequency power supply via an L−C matching network. The whole system was enclosed in a Faraday cage. Prior to each experiment the chamber was cleaned by scrubbing with detergent, rinsing in water, oven-drying, and finally running a 50 W continuous wave air plasma operating at 0.2 mbar pressure. Next, a sample of nonwoven polypropylene cloth (Corovin GmbH, 1.25 mm thick) or silicon (100) wafer (MEMC Materials Inc.) was placed into the reactor, followed by introduction of 4-vinyl pyridine vapor at 0.2 mbar and then the ignition of the electrical discharge (optimum duty cycle: P_p = 40 W, $t_{on} = 100\ \mu$s, and $t_{off} = 4$ ms) for 10 min duration. Upon plasma extinction, the monomer was allowed to continue to flow through the system for an additional 5 min in order to quench any remaining active sites. This gave a film thickness of 150 nm.

The pyridine functionalized surfaces were then immersed into a 10% (v/v) solution of bromobutane (Aldrich, +99%) mixed with propan-2-ol (Aldrich, + 99%). The reaction mixture was simply refluxed at 70 °C for periods of up to 8 h to yield poly(4-vinyl pyridine) quaternized surfaces, Scheme 1. The final stage of preparation comprised rinsing in methanol and distilled water prior to air-drying.

Film thickness measurements were carried out using a spectrophotometer (nkd-6000 Aquila Instruments Ltd.). Transmission−reflectance curves (over the 350−1000 nm wavelength ranges) were fitted to a Cauchy model for dielectric materials using a modified Levenberg−Marquardt method (48).

Table 1. XPS Atomic Percentages for Poly(4-Vinyl Pyridine) Prior to and Following Quaternization

	elemental composition (%)		
polymer	C(1s)	N(1s)	Br($3d_{5/2}$)
theoretical poly(4-vinyl pyridine)	87.5	12.5	
4-vinyl pyridine plasma polymer	87.0 ± 0.5	13.0 ± 0.5	
theoretical quaternized	84.6	7.7	7.7
plasma polymer-bromobutane (3 h)	85.0 ± 0.5	9.5 ± 0.5	5.5 ± 0.5
plasma polymer-bromobutane (6 h)	84.5 ± 0.5	7.0 ± 0.5	8.5 ± 0.5

A VG Escalab spectrometer equipped with an unmonochromatized Mg Kα X-ray source (1253.6 eV) and a concentric hemispherical analyzer were used for X-ray photoelectron spectroscopy (XPS) characterization of the functionalized surfaces. Elemental compositions were calculated using sensitivity (multiplication) factors derived from chemical standards, 1.00: 0.45:0.34:0.25 C(1s):-O(1s):N(1s):Br($3d_{5/2}$). All binding energies were referenced to the C(1s) hydrocarbon peak at 285.0 eV. The core level envelopes were fitted to a linear background and fixed-width-half-maximum (fwhm) Gaussian peak shapes (49) using a Marquardt minimization algorithm.

Fourier transform infrared (FTIR) analysis of the pulsed plasma polymerized films at each stage of reaction was carried out using a Perkin-Elmer Spectrum One spectrometer equipped with a liquid nitrogen cooled MCT detector. All spectra were averaged over 256 scans at a resolution of 4 cm^{-1}. Reflection−absorption (RAIRS) measurements utilized a variable angle accessory (Graseby Specac) fitted with a KRS-5 polarizer (to remove the s-polarized component) and set at 66°. In addition, substrate subtracted attenuated total reflectance (ATR) infrared spectra of N-alkylated plasma polymer films deposited onto polypropylene nonwoven cloth were acquired using a diamond ATR accessory (Graseby Specac Golden Gate).

Antimicrobial performance against *Staphylococcus aureus* (Gram positive) and *Klebsiella pneumoniae* (Gram negative) was monitored in accordance with the The Japanese Industrial Standard JIS Z 2801: 2000 Protocol Antimicrobial Products−Test for Antimicrobial Activity and Efficacy. (50) A predetermined volume of a 24 h culture of the relevant organism (microbes used in 0.1 M aqueous PBS buffer pH 7.0) was applied to functionalized polypropylene nonwoven cloth material for 24 h. Next, any residue of culture was swabbed off for resuspension in solution followed by plating out using appropriate agars (50 mL of a yeast/dextrose broth) and further incubated for 24 h at 35 °C. This procedure was repeated using uncoated nonwoven polypropylene cloth ("control" sample), and the number of colony-forming units on both cloths was measured by the viable cell-counting method (51). Surface antimicrobial activity of the treated cloths was determined by comparing results from the test sample to a simultaneously run control sample and expressed as a percentage reduction and colony-forming units (CFU) per milliliter (i.e., number of viable bacteria per milliliter).

3. RESULTS AND DISCUSSION

XPS analysis of the deposited 4-vinyl pyridine pulsed plasma polymer layer confirmed the presence of only carbon and nitrogen at the surface, with no Si(2p) signal detected from the underlying silicon substrate, Table 1. Furthermore, a good correlation was found to exist between the atomic percentages calculated for the monomer (theo-

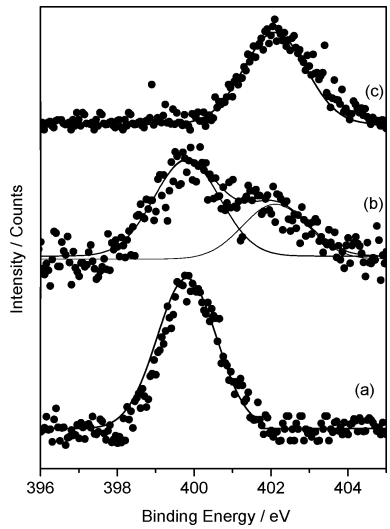


FIGURE 1. N(1s) XPS spectra of (a) pulsed plasma deposited poly(4-vinyl pyridine) film; (b) following quaternization reaction with bromobutane for 3 h; and (c) following quaternization reaction with bromobutane for 6 h.

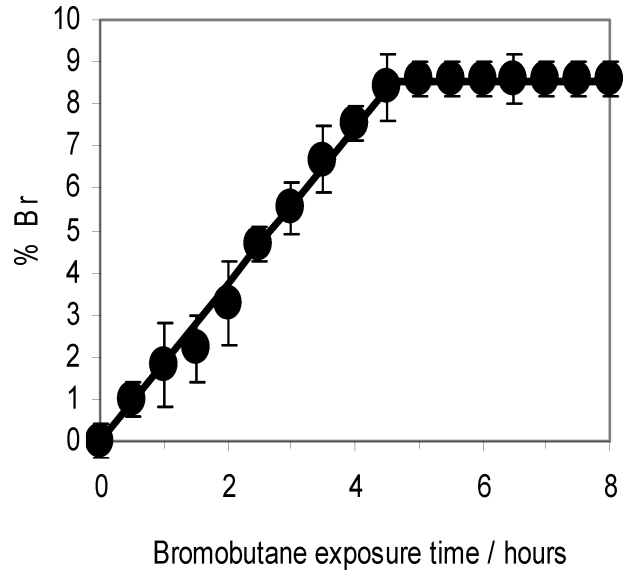


FIGURE 2. XPS bromine concentration at the surface of pulsed plasma deposited poly(4-vinyl pyridine) films following immersion in bromobutane solution and rinsing as a function of exposure time.

retical) and 4-vinyl pyridine plasma polymer film, thereby indicating that the majority of the deposited polymer backbone comprised pyridine units. This high level of structural retention is in marked contrast to high-power continuous wave plasma polymers derived from 4-vinyl pyridine (52, 53).

Following immersion of the pulsed plasma poly(4-vinyl pyridine) films in bromobutane quaternization solution, the appearance of a Br($3d_{5/2}$) signal at 68.8 eV corresponding to the presence of bromide counter-anions (54) indicated successful pyridine-ring quaternization, Scheme 1. This was accompanied by a corresponding shift in the N(1s) peak from 399.8 eV (neutral) to 402.1 eV (positively charged) (55), Figure 1. The extent of surface quaternization (% bromine) was found to correlate to the period of immersion in bromobutane solution until eventually complete quaternization (i.e., approximately 1:1 ratio of N(1s): Br($3d_{5/2}$) was reached, Figure 2. The present 100% quaternization is a significant improvement compared to earlier studies where between 30−90% conversions are reported (24, 27, 30).

The molecular structure of the plasma polymer coatings was probed by infrared spectroscopy, Figure 3. In the case of the 4-vinyl pyridine monomer, characteristic fingerprint features include (56, 57): vinyl C═C stretching (1624 cm^{-1}), aromatic C═C stretching (1603, 1585, and 1489 cm^{-1}), and C═N stretching (1414 cm^{-1}). Low-power pulsed plasma deposition of poly(4-vinyl pyridine) yielded all of the aforementioned bands, except those associated with the vinyl C═C feature (1624 cm^{-1}) which had undergone glow discharge initiated polymerization. In addition, a new band associated with CH_2 deformation (1453 cm^{-1}) was evident due to growth of the aliphatic polymer backbone. Exposure to bromobutane solution gave rise to the appearance of a sharp new band at 1640 cm^{-1} indicative of pyridine ring quaternization (58). This can be attributed to C═N semicircle stretching (usually at 1414 cm^{-1}) experiencing the electromeric effect imposed by quaternization and thus shifting to higher frequency (1640 cm^{-1}) (59).

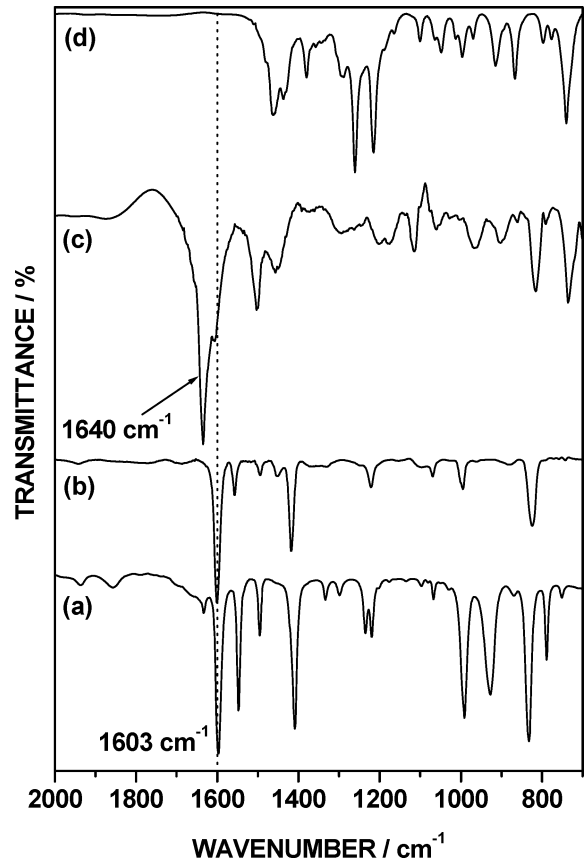


FIGURE 3. FTIR spectra of: (a) 4-vinyl pyridine monomer; (b) 4-vinyl pyridine pulsed plasma polymer; (c) 4-vinyl pyridine pulsed plasma polymer quaternized with bromobutane; and (d) bromobutane.

Antibacterial testing of the quaternized 4-vinyl pyridine plasma polymers, deposited onto a polypropylene nonwoven cloth, exhibited bactericidal activity of Gram-positive and Gram-negative bacterial colonies, Table 2. The level of antibacterial activity (R) is industrially classified as grade 1, 2, or 3 for Antibacterial Textiles (Grade 1, $R \geq 99.9\%$; grade

Table 2. Bacteria Counts for Bromobutane Quaternized 4-Vinyl Pyridine Pulsed Plasma Polymer Attached to Polypropylene Non-Woven Cloth

bacterium	gram type	bromobutane exposure time (h)	bacteria killed after 24 h (%)	log (CFU/mL) after 24 h
Staphylococcus aureus	positive	0	0	6.7
		3	83.9	5.9
		6	>99.9	2.3
Klebsiella pneumoniae	negative	0	0	6.6
		3	86.5	5.7
		6	>99.9	2.1

2, 99% < R < 99.9%; grade 3, 0% < R < 99%) (60). Cloths that had been fully quaternized led to complete sterilization of both forms of bacterial colonies on the surface (i.e., Grade 1, $R \geq 99.9\%$). Bacterial colony reduction yielded a Log Kill value (61, 62) of 4.4 (99.996%), which surpasses bactericidal activity data (Log Kill value of 3 (99.9%)) from earlier studies relating to quaternized surface pyridinium groups (23−25, 29, 30). Also, the minimal clinical standard criterion (Log Kill >3) set by the Clinical and Laboratory Standards Institute (CLSI) is met (61).

Other coated substrate materials such as glass and steel were also found to exhibit Log Kill values greater than 4. In all cases, excellent adhesion (absence of delamination) of the functional layers was observed despite the quaternization step being carried out under reflux conditions at 70 °C for periods up to 8 h. For silicon and glass substrates, Si−C bonds will be responsible for adhesion (63); similarly, M−C bonds will be responsible for adhesion in metals (64), whereas for polymers, it is free radical sites created by the electrical discharge (65). These bioactive surfaces could be easily regenerated by rinsing in water (which dislodges the dead cell debris).

Variations of this approach such as atomized spray plasma deposition (66) (ASPD) also lead effective to bioactive coatings. Alternative precursors for the plasma deposition could include diallyldimethylamine (67), 4-vinylaniline (68), and *N*,*N*-dimethyldodecylamine (69), these are all capable of quaternization to give immobilized bactericidal ammonium-type salt moieties. The ability to kill *Staphylococcus aureus* is promising because it is a leading cause of nosocomial infections (antibiotic-resistant strains of bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA)) and nuisance organisms such as those which produce ammonia from urine (70).

4. CONCLUSIONS

Pulsed plasma polymerization of 4-vinyl pyridine provides a substrate-independent route for producing structurally well-defined pyridine functionalized solid surfaces. These immobilized pyridine centers can be quaternized with bromoalkane solution to generate bioactive surfaces which are bactericidal toward *Staphylococcus aureus* (Gram positive) and *Klebsiella pneumoniae* (Gram negative) micro-organisms. Regeneration of antibacterial activity can be accomplished by simply rinsing in water to dislodge the killed biological species.

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