Articles

Synergistic Activity of Hydrophilic Modification in Antibiotic Polymers

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Quaternized poly(vinylpyridine) is known to kill up to 99% of drug-resistant Gram-positive and -negative bacteria but shows minimal biocompatibility. We report enhanced bactericidal activity of vinylpyridine through copolymerization with hydroxyethyl methacrylate and poly(ethylene gycol) methyl ether methacrylate. Copolymers with increasing comonomer content were synthesized by radical polymerization and quaternized with hexylbromide. We assessed the effects of the changes in polymer composition on the bactericidal activity of the surface activity using a bioluminescent pathogenic strain of *Escherichia coli* (O157:H7). By recording the photoluminescence emitted by these bacteria in contact with the copolymers, it was shown that several of the copolymers possess better antibacterial efficiency than quaternized poly(vinylpyridine). Results indicate that several of the copolymers synthesized possess antibacterial activity ∼20 times greater than the pure quaternized poly(vinylpyridine) homopolymer, while only containing 1 wt % hexylated pyridinium. This behavior is explained by the increased surface wettability of the copolymers containing lesser amounts of poly(vinylpyridine), as bactericidal behavior correlates to the hydrophilicity of the system as measured by contact angles. A hydrophilicity based design−paradigm can significantly improve both the efficacy and the biocompatibility of antibacterial materials.

Introduction

Tens of thousands of people die in the United States each year from material-facilitated nosocomial infections such as those associated with burns and catheters. Materials with broad spectrum anti-bacterial properties are necessary to improve upon current technology and stay ahead of drug-resistant bacteria.[1−3] Bactericidal properties can prevent the proliferation of harmful micro-organisms and reduce or eliminate the need for antiseptic treatments on surfaces people contact regularly. Pathogens can easily be spread through contact, and materials that respond by preventing growth and viability of bacteria would significantly reduce exposure to humans. Bactericidal materials could prevent the spread of many diseases and even offer a reply to the growing threat of biological terrorism and warfare.

Previous work to produce bactericidal materials by incorporation of a leaching bioactive agent into a material[4−7] causes drawbacks due to environmental threat, short-term killing efficacy, and inability to attack airborne bacteria. Therefore, research has recently focused on inherently bactericidal materials. Cationic polymers, such as polyammonium coatings, were proven to be efficient biocides.[8−11] Most recently, it was shown that alkylated pyridinium and ammonium polymers, such as poly(4-vinyl-*N*-alkylpyridinium bromide) covalently attached to glass slides, could create a surface that killed airborne and waterborne bacteria on contact.[12,13] A variety of drug-resistant pathogens are affected by poly(vinyl-*N*-hexylpyridinium bromide), including methicillin- and penicillin-resistant bacteria.[14] These antibacterial moieties have also been attached to the surface of common woven textiles such as cotton, wool, nylon, and polyester.[15,16] Still, applications remain severely limited by the poor solubility in water and poor biocompatibility of poly(vinylpyridine) materials,[17,18] specifically their tendency to cause irritation to skin.[19]

The purpose of this work was to design copolymers possessing bactericidal properties with improved hydrophilicity and biocompatibility. We synthesized copolymers based on poly(4-vinylpyridine) (PVP), a polymer known for its bactericidal activity once quaternized. Copolymers were prepared with monomers known to be strongly hydrophilic and biocompatible: hydroxyethyl methacrylate (HEMA) and polyethylene glycol methyl ether methacrylate (PEGMA).[20] The goal was to optimize anti-bacterial properties while improving solubility and biocompatibility.

Experimental Procedures

Copolymer Synthesis. VP, PEGMA, and HEMA (Aldrich, St. Louis) were purified by means of either a vacuum trap-to-trap distillation (HEMA and VP) or column chromatography eluted from chloroform (PEGMA). Copolymers were synthesized by radical copolymerization with 2,2′-azobis-isobutyronitrile (AIBN) as initiator (Scheme 1). The reactants were stirred at 70 °C for 48 h under flowing N_2 to prevent oxidation. As the monomer contents were varied, the AIBN proportion was held constant to a mass ratio of 22:1 monomers/AIBN. To investigate the effects of hydrophilicity on behavior, various compositions of VP with HEMA and PEGMA were synthesized, and unless noted otherwise, molar ratios are quoted. Further characterization techniques (FTIR, NMR, and GPC) were conducted and confirmed the

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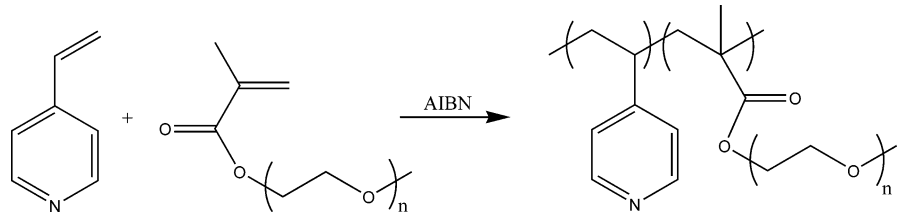
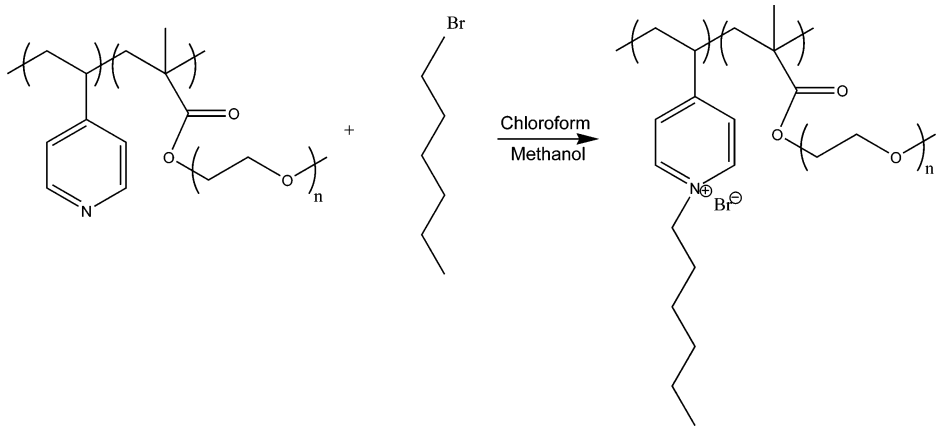
Scheme 1. Synthesis of Poly(VP-*co*-PEGMA)



Scheme 2. Quaternization of Poly(VP-*co*-PEGMA)



synthesis but are not reported here. Polymers were in the range of 20 000 to 50 000 g/mol molecular weight. Reactivity ratios were approximately 1 as determined by Fineman−Ross and Kelen−Tudos of the NMR data, yet the polymers were depleted in hydrophilizing monomer (HEMA or PEGMA).

Quaternization. Copolymers were quaternized with a 3-fold excess of HB in a mixture of chloroform and methanol by reflux at 70 °C for 48 h (Scheme 2). They were precipitated in hexane, recovered, and dried under vacuum. Complete alkylation was determined by infrared spectroscopy. A peak occurs at 1600 cm^{-1} that is indicative of the pyridinyl ring and incurs a peak shift to 1650 cm^{-1} upon quaternization. Relative sizes of 1600 and 1650 cm^{-1} indicate the degree of quaternization. Alkylation was considered complete if the sample was found to have complete attenuation of the 1600 cm^{-1} band.

Contact Angle. Contact angle measurements were performed on copolymer films prepared by casting onto glass microscope slides from chloroform and methanol (90:10 v/v). All measurements were obtained on a Rame-Hart Advanced Goniometer. PEGMA series copolymers are water soluble below 90% VP, preventing measurement of polymers below this critical amount of VP.

Bacterial Testing. Polymer samples were cast onto slides as stated before. Bacteria were grown in a minimal media utilizing glucose as a sole carbon and energy source. One hundred microliter aliquots of the *Escherichia coli* O157:H7 rendered bioluminescent using a lux luciferase system were taken from a culture grown to an OD_{600} of 1.0 (0.1 mL corresponds to approximately 10^8 cells) and placed in contact with the coated slides. The intensity of the bioluminescence (photons per second) was recorded as a function of time for 2 h using a photomultiplier tube. The bacteria were not initially media inhibited, so show lag-log behavior of intensity from the outset. Bactericidal surfaces were plated with traditional methods to ensure that bioluminescence attenuation corresponded to bacterial death.

Results and Discussion

Assays used to determine effective concentrations of disinfectants for sanitizing conditions typically implement plate count methodology to detect surviving bacteria. Plate count methodologies can be labor intensive, are time-consuming, and require days for detection. Bioluminescence is a superb monitoring tool with excellent sensitivity and limits of detection. Since bioluminescence does not persist after cell death, bioluminescence is an attractive method for in situ real-time measurement of bacterial numbers and cell viability. In addition, while traditional colony counting techniques are excellent at the measurement of bacteristatic surfaces, they have difficulty with truly bactericidal surfaces. To be detected, the bacteria must grow and form viable colonies, the numbers of which are compared to controls. Surfaces that reduce bacterial count fast enough to prevent stable colonization provide difficulties with these techniques. Bioluminescence reporter methods do not have these limitations and are ideally suited for the fast screening of large numbers of materials reported here.

Bactericidal efficiency of quaternized copolymers with VP was measured using a bioluminescent *E. coli* O157H:7. The glass control (Figure 1b) exhibits an initial increase of intensity due to the increased availability of oxygen; however, the intensity begins to decrease as the lack of water and nutrients causes bacterial death (Figure 1). PVP−-HB (Figure 1a),

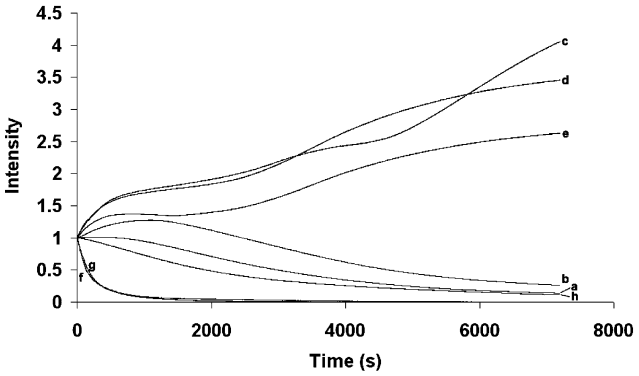


Figure 1. Time-dependent bioluminescence intensity of the bioluminescent *E. coli* O157:H7 deposited onto N-hexylated copolymers of 4-vinyl pyridine (VP) and hydroxyethyl methacrylate (HEMA). (a) PVP-HB, (b) control, and (c) PHEMA, copolymers with VP to HEMA mol ratios of (d) 10:90, (e) 25:75, (f) 90:10, (g) 95:5, and (h) 99:1. Zero time intensities are normalized to unity.

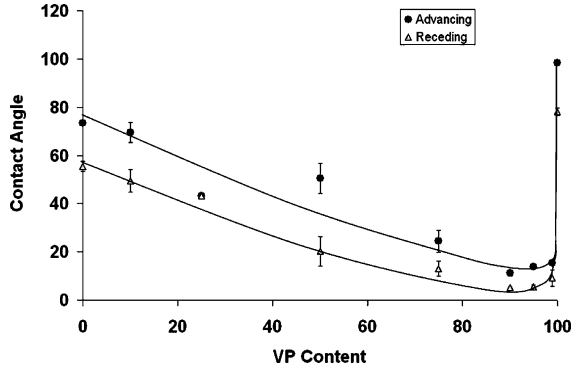


Figure 2. Advancing (filled circles) and receding (open triangles) water contact angle measurements for dry, vitreous N-hexylated poly(4-vinylpyridine-*co*-hydroxyethyl methacrylate) copolymers (P(VP-*co*-HEMA)-HB) as a function of 4-vinyl pyridine content (line added to guide the eye).

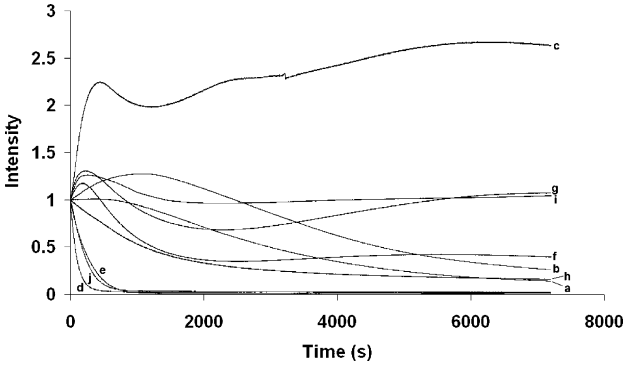


Figure 3. Time-dependent bioluminescence intensity the bioluminescent *E. coli* O157:H7 deposited onto N-hexylated copolymers of 4-vinyl pyridine (VP) and poly(ethylene gycol) methyl ether methacrylate, MW ~1100 g/mol (PEGMA1100). (a) PVP−-HB, (b) control, and (c) PPEGMA1100, copolymers with VP to PEGMA1100 mol ratios of (d) 10:90, (e) 25:75, (f) 50:50, (g) 75:25, (h) 90:10, (i) 95:5, and (j) 99:1. Zero time intensities are normalized to unity.

prevents the initial light increase, and the bioluminescence decreases over time relative to the control. However, the bioluminescence does not completely attenuate over the 2 h test.

Copolymers of VP and HEMA (Figure 1) displayed improved bacteria killing over PVP−-HB. As the fraction of HEMA is increased in the copolymer, the materials become more bactericidal up to an optimum mol ratio, between 90:10 (Figure 1f) and 95:5 (Figure 1g). Both copolymers achieved 50% reduction in luminescence ~10 times faster than the homopolymer PVP−-HB. Both copolymers attained total attenuation (signal intensity within the noise) within an hour. P(HEMA) (Figure 1c) showed lag−log growth behavior and a nearly 4-fold increase in luminescence over the 2 h test. Following a rule-of-mixtures behavior, P(VP-*co*-HEMA)-HB with monomer mol ratios of 25:75 (Figure 1e) and 10:90 (Figure 1d) showed behavior intermediate between P(HEMA) and PVP−-HB, with 25:75 being the more effective formulation. Anomalously, the 99:1 mol ratio (Figure 1h) was intermediate in performance between the best samples and the pure PVP−-HB, indicating that there is an optimum mol ratio of VP to HEMA.

The increase in antimicrobial activity as HEMA is added to the formulation can be attributed to the enhanced wettability of the materials. Having pendent hydroxyl functionality gives HEMA the ability to interact with water to a much greater degree than the hydrophobic alkyl chains in the quaternized PVP. Observations showed that cells act in accordance with the principles of surface energy, spreading on high energy hydrophilic surfaces and beading on low energy hydrophobic surfaces.[21] As HEMA is incorporated, the surface energy of the material increases (more hydrophilic), causing the bacteria to spread and come into contact with more of the copolymer surface and thereby come into contact with more bactericide. With increasing HEMA concentration, gains in surface energy cannot overcome the diminishing amount of active monomer in the bactericide, and the increased performance reverses−−adding more HEMA degrades the ability of the copolymer to kill bacteria. Dynamic water contact angle behavior of the VP/HEMA copolymers was measured as a function of molar percentage of VP (Figure 2). Low amounts of HEMA in the copolymer showed a dramatic influence on the contact angle behavior. Adding 1% HEMA into the formulation causes a drop of almost 80° in advancing the contact angle. Hydrophilicity varies as 90:10 > 95:5 > 99:1 > 100:0 (pure PVP). The order coincides with the bactericidal efficacy. The most wettable surface occurred at the 90:10 point and corresponded to the most effective formulation, although 95:5 was close in behavior in both tests as well. At greater than 10 mol % HEMA contents, the surface energy started a nearly monotonic increase in hydrophobicity until pure vitreous HEMA was reached. The decreased water wettability and the decreased active ingredient are likely both detrimental to bactericidal performance, but it is impossible to quantify the degree to which each effect acts, due also to the fact that wetting behavior does not follow the rule-of-mixtures. It is possible that devitrification of the material or swelling kinetics play some role. Dynamic water contact angle of the PEGMA1100 and PEGMA300 series copolymers was performed. However, due to water solubility of all PEGMA copolymers below 90% VP content, the data set is limited to three points each. In each case, the data were consistent with the HEMA results with the most wettable formulation being the most active of those tested.

To further improve wettability, VP was copolymerized with PEGMA in the hope that the water soluble PEG side chain would increase interaction with water. Quaternized copolymers of VP and PEGMA1100 (molecular weight of 1100 g/mol) demonstrated the most significant improvements in antibacterial properties of all copolymers seen in the study with ratios (VP/PEGMA1100) of 10:90 (Figure 3d), 25:75 (Figure 3e) and 99:1 (Figure 3j). Pure P(PEGMA1100) (Figure 3c) supported bacteria growth with an approximately 2.5-fold increase in bioluminescence intensity after 7200 s (2 h). Although copolymer 50:50 (Figure 3f) is bactericidal, this formulation was not as effective as the higher molar ratios. The bioluminescence decreased for the first 2400 s (40 min), followed by a gradual increase to a level above the control (Figure 3b). Molar ratios 75:25 (Figure 3g) and 95:5 (Figure 3i) are bacteriostatic−−showing the same bioluminescent activity after 7200 s (2 h) as at the beginning. Copolymer 90:10 (Figure 3h) showed improved bactericidal behavior being just as effective as PVP−-HB (Figure 3a), yet faster. The last copolymer formulation with the highest concentration of VP has a high activity reducing bioluminescence activity in a manner similar to 25:75 (Figure 3e) and is barely distinguishable from it.

While water solubility limited most contact angle analysis to three points, contact angle behavior was consistent with HEMA results. P(VP-*co*-PEGMA1100) 99:1 was the most active PEGMA1100 polymer and the most wettable as the advancing contact angle was reduced to approximately 60°.

The bioluminescence intensity measured for PEGMA300 (molecular weight of 300 g/mol) copolymers displayed limited improvement in bactericidal properties (Figure 4). PPEGMA300 (Figure 4c) supported bacterial growth with luminescent activity

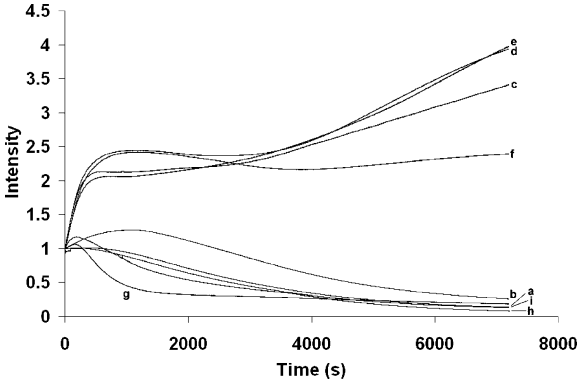


Figure 4. Time-dependent bioluminescence intensity of the bioluminescent *E. coli* O157:H7 deposited onto N-hexylated copolymers of 4-vinyl pyridine (VP) and poly(ethylene gycol) methyl ether methacrylate (PEGMA300). (a) PVP−-HB, (b) control, and (c) PPEGMA300, copolymers with VP to PEGMA300 mol ratios of (d) 10:90, (e) 25:75, (f) 50:50, (g) 75:25, (h) 90:10, and (i) 99:1. Zero time intensities are normalized to unity.

∼3 times higher after 7200 s (2 h). Overall, the P(VP-*co*-PEGMA300) copolymers showed similar behavior to the rule-of-mixtures with the lowest concentration VP copolymers of mol ratios 10:90 (Figure 3d) and 25:75 (Figure 3e) showing similar behavior to pure P(PEGMA300). These copolymers supported bacterial growth well and did not show antibacterial behavior. Copolymer 50:50 (Figure 3f) reduced bioluminescence relative to P(PEGMA300); however, the formulation still supported growth. Copolymers with VP/PEGMA300 ratios of 75:25 (Figure 4g), 90:10 (Figure 4h), and 99:1 (Figure 4i) all showed similar bactericidal properties when compared to PVP−-HB (Figure 4a) with approximately 25% bioluminescence remaining after 7200 s (2 h). The mol ratio 75:25 (Figure 4g) had the fastest initial decrease, while 90:10 (Figure 4h) attained the lowest signal, yet differences may be due to the bacteria being in different growth stages at the beginning of the test. Again, water solubility limited water contact angle results. Furthermore, the lack of differentiation among the high VP containing polymer activity data prevented analysis in this regard.

In contrast to HEMA and PEGMA300 copolymers (Figures 1 and 4), P(VP-*co*-PEGMA1100) copolymers with the lowest VP content: 10:90 and 25:75 showed drastically improved bactericidal behavior being two of the best samples tested. Monomer ratios of 10:90 and 99:1 represent two ends of the composition spectrum, yet both demonstrated extremely high bactericidal efficiency. The high VP content end is likely dominated by the same wettability concerns as the HEMA copolymers. Like HEMA, PEGMA1100 is a monomer with good hydrophilicity, and so copolymers should help bacteria spread and cause greater contact with the bactericidal material. At 90% PEGMA and below, the materials had such a high PEGMA1100 content that the polymers became water soluble. In practice, this means that the methodology we used for surface testing of bacterial vitality breaks down. As an aqueous aliquot of bacterial culture is placed on the slide, the copolymer is likely to dissolve or swell, surrounding the bacteria with polymer. This dissolution greatly increases bacterial contact with the bactericidal surface, complicating the results. Dissolution kinetics further complicates analysis, and so it is impossible to make more than broad statements about the data set. While swelling and dissolution cannot be ruled out as a root cause of the greatly increased performance of the polymers, it seems unlikely that it is the only cause of such a large increase in performance. Copolymer 10:90 reduced intensity to 50% in 120 s with complete attenuation in 900 s (15 min). Copolymer 25:75 reduced luminescence intensity to 50% in 360 s and attained attenuation in 1500 s (25 min). Regardless, these materials are the most active of the materials, with the ability to kill 20 times faster than quaternized PVP. Considering that 10 M % equates to 1 wt % of active moiety in the polymer, the behavior of this formulation is extreme.

In contrast to HEMA copolymers and PEGMA1100 copolymers, no formulation of PEGMA300 and VP attained complete attenuation of bioluminescence for reasons that are not fully understood. The behavior is unlikely to be entirely controlled by dissolution/swelling, as high PEGMA300 content materials do not show the same bactericidal behavior as PEGMA1100 materials. As well, PEGMA300 materials are unlikely to have very dissimilar wetting behavior as PEGMA1100 materials. However, while data are sparse due to water solubility of most of the PEGMA polymers, the water insoluble polymers mostly support this view. A possible explanation arises when it is considered that PEG is commonly used to biocompatibilize surfaces and prevent protein adsorption.[22] Protein adsorption is key to controlling the appropriate body response and to render a material biocompatible. Evidence has shown that longer PEG chains can do this more effectively up to a limit.[21] Hydrophobicity prevents cell spreading but also can cause large-scale protein adsorption.[22,23] For the surfaces presented here, protein adsorption would have a detrimental effect as the protein would occlude the active sites (the alkylated pyridinium) preventing their action on the bacteria. Lower protein adsorption should then lead to higher activity. Results from PEGMA300 and PEGMA1100 are consistent with this hypothesis; however, no supporting data on protein adsorption are currently available. We must state that even if our hypothesis is true, we make no claim as to the biocompatibility of these materials; our argument is only as one possible explanation for the observed behavior.

Conclusion

These results show that copolymers of inherently bactericidal monomers with inactive diluent monomers significantly enhance antibacterial behavior in a synergistic fashion. At the same time, hydrophilicity and solubility were increased. Optimal formulations were up to 20 times better than quaternized PVP alone using a minimal (∼1 wt %) active moiety. Water contact angle measurements of P(VP-*co*-HEMA)-HB show that bactericidal efficacy correlates to water wettability, explaining the synergistic role of the hydrophilic monomers. As such, maximizing the water contact angle should be a primary design criterion for the creation of bactericidal materials.

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