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Antibacterial Properties of a Cationic Imidazolium-Containing Poly(Ionic Liquid)

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ANTIBACTERIAL PROPERTIES OF A CATIONIC IMIDAZOLIUM-CONTAINING POLY(IONIC LIQUID)

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ABSTRACT

Poly(ionic liquids), or PILs, have been shown to have a number of biological applications as ligands, drug delivery vehicles, and nonviral DNA delivery mechanisms. Additionally, PILs have been proven to be efficient antibacterials. Imidazolium-containing PILs have promising results in antibacterial studies, but relating to their chain and charge density, only the effects of mono- & bis-imidazolium PILs have been sufficiently described in literature. The work detailed in this thesis aimed to further existing research on the effect of chain density by controlling the UV-initiator immobilization time. The effects of chain density were investigated by grafting poly(vinylimidazolium chloride) onto SiO_2 glass substrates with varying initiator immobilization times. Substrates were tested against *Escherichia coli (E. coli)* for 24 h. The total reduction was quantified using a viable cell count. Results showed up to a 67.8% reduction in viable *E. coli* with one 120 min UV-initiator immobilization time sample. The average percent viable reduction among all conditions was determined to be statistically different by standard error compared to the control (52.3 ± 6.1 % vs. 0.0 ± 7.3 %, respectively). However, there was no general trend, increasing or decreasing, relating to UV initiator immobilization time on antibacterial performance. Possible reasons for this were the volume of bacteria tested with each substrate, time of incubation, or UV-initiator quality. More research is needed to accurately determine whether UV-initiator immobilization times, attributing to chain density, have a significant effect on antibacterial performance.

INTRODUCTION

Adhesion of bacteria onto a surface can cause a multitude of problems in areas such as healthcare, food and beverage storage, water treatment, and the shipping industry. (1) It has been reported that around 64% of all infections acquired while a patient was in the hospital are a direct result of growth of viable bacteria on a medical device. Besides infections, the buildup of these organisms, known as biofilm formation or biofouling, can affect industry processes by reducing flow rate and causing premature degradation of materials that make up pipes and vessels. Issues like these can compromise wastewater treatment or pharmaceutical production processes. (2) The use of antibiotics to circumvent the problems mentioned here can be used but many times are ineffective due to antibacterial resistance. Thus, the need for novel antibacterial coatings and surfaces is of extreme importance. (3)

Polymer brushes grafted either to or from a surface have been an area of extensive focus in relation to their antibacterial activity. While both have shown antibacterial activity, the grafted from method has been shown to have a more even distribution of charge over the modified surface, which is important when looking at antibacterial properties. In addition, brushes containing both polycations and polyanions have been investigated. Polycations show high antibacterial capacity, while polyanions allow for reduction of bacterial adhesion initially and can affect biofilm formation architecture over time. This in turn allows for the biofilm to be more easily removed. (1)

A particular type of polymer brush that has potential to be an efficient antibacterial are poly(ionic liquids), or PILs. PILs are a type of polyelectrolyte that contain repeating units of an inorganic anion and organic cation that are or can be ionized, contributing to their unique chemical and

physical characteristics. (4,5) Several types of PIL-based membranes synthesized from pyrrolidinium, quaternary ammonium, and imidazolium cationic moieties have been reported as effective antibacterials against both gram negative and positive bacteria. (6-8) In the case of many of the cationic PILs, the mechanism of action occurs through disruption of the cell membrane causing cell contents to leach out or through inhibition of fatty acid synthesis. (5,9)

In particular, due to their high cationic charge density, imidazolium-containing PILs have been noted to have a variety of biological applications as ligands, drug delivery vehicles, and nonviral DNA delivery mechanisms, and previously-mentioned antibacterials. (10) While imidazolium-based PILs have been intensely studied for a variety of biological applications, the exact conditions that optimize antibacterial performance are hard to pin point due to strong dependence on both the cationic and anionic moieties as well as the overall charge density. The effects of charge density of the imidazolium group have only been studied in mono- and bis-imidazolium PILs, leaving the effects of imidazolium charge density largely unstudied. (5)

In the method of PIL grafting used in this study, imidazolium chain density, thus charge density, is controlled by the time allowed for deposition of the UV initiator. (11) Therefore, to determine the effects of charge density in imidazolium-containing PILs on antibacterial activity, UV initiator immobilization time was varied.

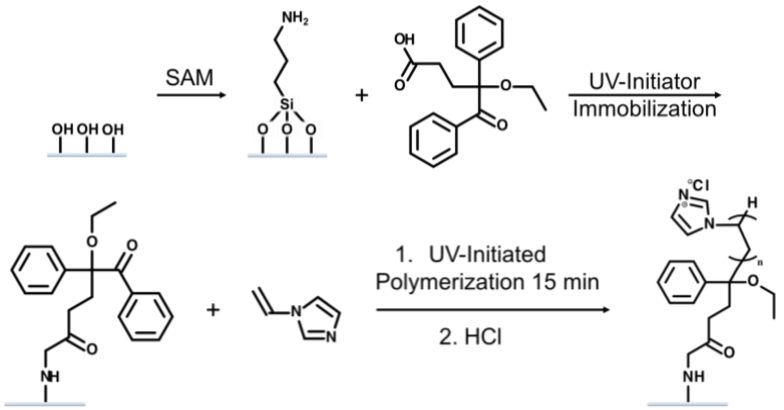
MATERIALS AND METHODS

Purified water (.06 mS/cm) was collected through a combination Water Pro/RO reverse osmosis and Pro Plus deionization purification system by Labconco Corp. (Kansas City, MO). 1-vinyl imidazole (99%) was purchased from Alfa Aesar (Ward Hill, MA). 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide (EDC, 97%), 3-aminopropyl-triethoxysilane (APTES, 98%), benzoin ethyl ether (BEE, 97%), Dulbecco's phosphate buffered saline (PBS), and agar were all purchased from Sigma Aldrich (St. Louise, MO). Ethanol (100%), sulfuric acid (H_2SO_4, 95%), hydrogen peroxide (H_2O_2, 30%), and 2xYT broth were purchased from VWR (West Chester, PA). Hydrochloric acid (HCl, 36.5–38% w/w) was purchased from EMD Millipore (Darmstadt, Germany). *Escherichia coli* (Migula) Castellani and Chalmers (*E. coli*, ATCC 13706) was purchased from ATCC (Manassas, VA). No further purification was done before use of all chemicals. Glass substrates were standard 1.5 cm x 1.5 cm SiO_2 microscope slides.

Poly(Vinyl Imidazole) Substrate Modification

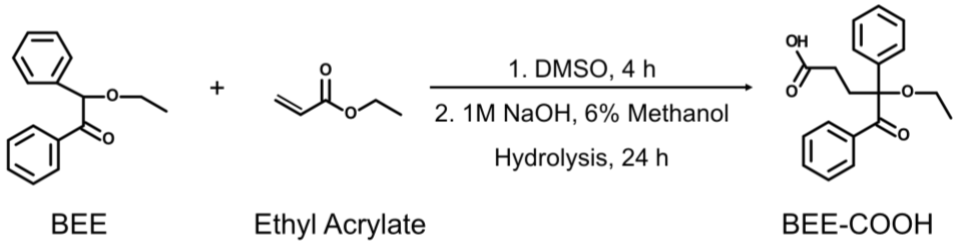
The surface modification of SiO_2 glass chips can be summarized in three distinct steps (Scheme 1). In preparation for the modification, the UV initiator was synthesized and organic residues were removed from the glass surface. The removal of organic acid residues also causes formation of hydroxide groups on the substrate. The first step in the overall grafting of poly(vinyl imidazole) was the addition of a self-assembled mono (SAM) layer with functionalized amino groups. After the SAM layer was added, the UV-initiator was immobilized onto the surface for either 30, 60, 90, or 120 min. The final step was the UV-initiated free radical polymerization of vinyl imidazole onto the glass surface followed by submersion in HCl to generate the final grafted PIL: poly(vinylimidazolium chloride) or PVImCl.



Scheme 1. *Overall reaction for grafting of PVImCl onto SiO_2 glass substrate.*

Synthesis of UV Initiator

The synthesis of the UV initiator (BEE-COOH) was previously synthesized as described by Qian et al. from the starting material of benzoin ethyl ether (BEE). (11) The overall reaction scheme for synthesis can be seen in Scheme 2.



Scheme 2. *Synthesis of UV initiator, BEE-COOH.*

Addition of Hydroxyl Groups

To remove organic residues and form reactive hydroxide groups on the glass chip surface, glass chips were treated with heated piranha solution. The oxidative solution was prepared by mixing 70% (v/v) sulfuric acid with 30% (v/v) hydrogen peroxide at a ratio of 3:1 H_2SO_4:H_2O_2 and heating to 50˚C. All substrates were submerged in the heated piranha solution and allowed to soak for 2 h. After 2 h, chips were washed 3 times with DI water followed by washing with 100% ethanol 3 times.

Formation of APTES SAM layer

Glass chips were submerged in a well-mixed solution of 10mL 100% ethanol and 100 μL 3-aminopropyl-triethoxysilane (APTES). After being shaken for 1 h, substrates were cured at 135˚C for 10 min. Glass chips were then dipped in same SAM solution and cured as before three times for a total of four cured layers.

UV initiator immobilization

Prior to PIL chain synthesis, the UV initiator was immobilized on the glass chips containing functional amino groups as a result of the added SAM layer. The substrates were added to a flask with 10 mL DI water, 5 mg EDC, and 160 mg BEE-COOH. The container was sealed and placed on a shaker for the chosen immobilization time (30, 60, 90, or 120 min). The various immobilization times will have a proportional effect on PIL chain density which could affect the charge density and antibacterial performance. After the immobilization period, the substrates were rinsed with DI water three times and washed with water for 1 h.

PIL nanostructure synthesis via UV-initiated polymerization

Once the UV initiator immobilization was performed, UV-initiated free radical polymerization was utilized to graft poly(vinyl imidazole) chains to the substrate surface. A solution of 10 mL 1-vinyl imidazole (VI) monomer (100 g / L of water) was degassed in argon for at least 30 min prior to polymerization. Glass chips were then submerged in the degassed solution and placed in the UV reactor. Substrates were UV irradiated for 15 min followed by a 3-time wash with DI water. To generate a charge on the modified glass chips, all samples were placed in 12 N HCl for 24 h prior to use.

Antibacterial Testing and Quantification

To test glass chip antibacterial performance, cultures of *E. coli* were grown in 2xYT Broth until the mid-exponential growth phase was reached (OD_{600} of 0.6). Cultures were then centrifuged at 5000 rpm for 5 min after which the supernatant was decanted. The pellet was resuspended in sterile phosphate-buffered saline (PBS). Once resuspended, 1 mL of *E. coli* in PBS was incubated with one glass chip in a petri dish at 4˚C for 24 h. A control chip (no modification) was incubated in the same manner.

After the incubation period, the concentration of *E. coli* was determined using the viable cell count method in colony forming units (CFUs) per mL. For this procedure, 10-fold serial dilutions were made in duplicates for each sample. To do this, 200 μL of the *E. coli* sample was inoculated in the first well of a 48-well plate. 100 μL was transferred into the second well containing 900 μL of PBS. This step was repeated until a total of 10 serial dilutions were made. From each dilution, 100 μL of the *E. coli*/PBS solution was transferred to a nutrient agar plate (31 g/L 2XYT broth and 15 g/L agar). The samples were spread on each plate and incubated upside down for 12 h at 37˚C. At the end of the 12 h, 3 countable plates were chosen to include in the calculation of *E. coli* concentration, countable being no less than 25 or more than 250 CFUs. (12) The number of CFUs counted were used to calculate the final concentration as described in "Results".

RESULTS AND DISCUSSION

After the viable cell plate count, there were 3 to 4 countable plates from each sample. Three were counted and compared to the CFUs counted in the control. Qualitatively, the difference in the

number of CFUs could easily be seen by comparing a control plate to one of the samples. An example of this visual reduction can be found in Figure 1.

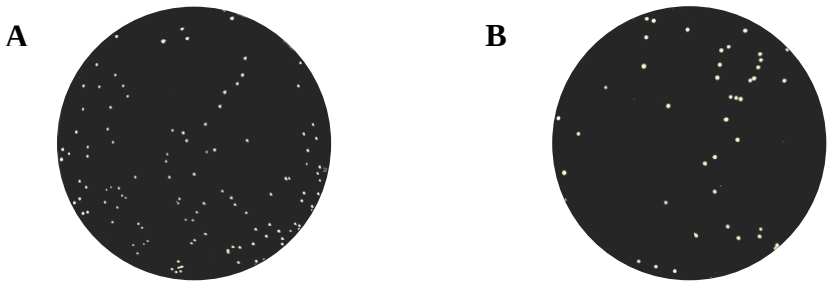


Figure 1. *Visual comparison* of CFUs on agar plate after incubation of (A) the control and (B) 120 min UV initiator immobilization condition.*

**CFUs were masked out (white) to enhance visual quality, but CFUs were not counted from digitally altered images*

The final *E. coli* concentration was calculated from the number of counted CFUs using the following equation:

$$\frac{CFU}{mL} = \frac{Number\ of\ CFU \times Dilution\ Factor}{Volume\ Plated}$$

After concentration was calculated, the results for each condition were averaged to find the final average concentration for each condition. Standard error was also calculated. Due to the nature of the experiment, the percent reduction of viable *E. coli* in percent CFU/mL compared to the control was used as a more meaningful indicator of antibacterial performance versus total reduction in CFU/mL. This percentage of reduction was calculated by taking the difference in concentration between the control/tested condition and then dividing by the control concentration. Results from this calculation can be found in both Table 1 and Figure 2. An example of this percent reduction calculation, the particular plate in Figure 1 (120 min UV-initiator immobilization time) had a 67.8% reduction in viable *E. coli* compared to the control.

Table 1. *Average total reduction and standard error in percentage of CFU/mL compared to the control concentration for each UV initiator immobilization condition.*

UV Initiator Immobilization Time (min)	Average Total Reduction (Percentage of CFU/mL)	Standard Error (Percentage of CFU/mL)
30	49.42 %	3.17%
60	54.28 %	8.60%
90	49.74 %	8.61%
120	55.85 %	4.08%

There was no general trend related to the effects of UV initiator immobilization times on percent reduction of viable *E. coli*. However, in general, all conditions did dramatically reduce the amount of viable *E. coli* compared to the control. On average, there was a 52.3 ± 6.1 % reduction in the percentage of viable *E. coli* compared to the control (0.0 ± 7.3 %).

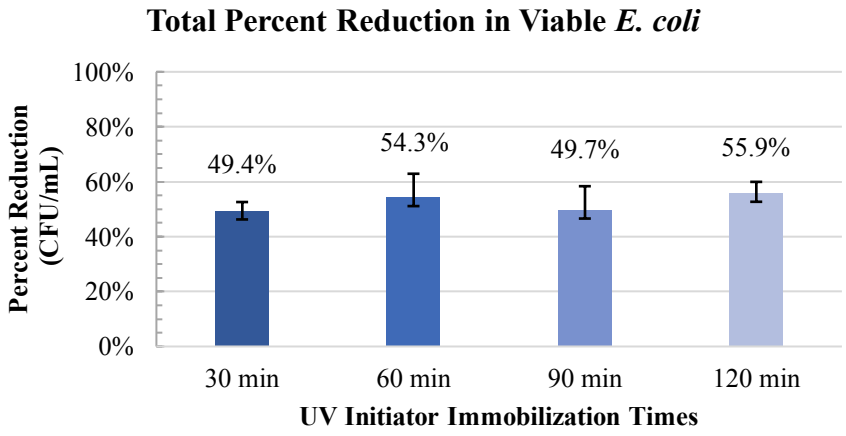


Figure 2. *Effects of Poly(1-Vinyl Imidazole) Chain Density on Amount of Viable E. Coli in percent reduction of CFU / mL.*

It was expected that the degree of chain density (controlled by UV-initiator immobilization time) would have had a significant effect on antibacterial activity, but results showed no trend of difference between the four immobilization times. Past research between mono and bis-imidazolium PILs has shown significant differences, suggesting that charge, thus chain, density

should have significant differences between each condition. (5) One explanation for this despite suggestions of importance in literature could be the quality of BEE-COOH. The initiator over time can lose performance capabilities if exposed to light or humidity, so possibly the UV initiator was not working at its optimal capacity. More likely, however, is that the volume of *E. coli* used to test antibacterial activity was too large or the incubation period was too long. Previous studies have used volumes as small as 100 μL to incubate on top of the substrate compared to the 1 mL that was used in this study, and incubation times have ranged from 2 to 24 h. Perhaps with a smaller volume or time of incubation, any significant differences between the initiator immobilization times would be more apparent. Additionally, studies using smaller volumes to test viable *E. coli* reduction saw almost 100% reduction. In comparison, the average reduction in this study was 52.3 ± 6.1 %. However, because of the wide range of conditions used in literature, it is difficult to determine whether this result is due to specific incubation conditions or the overall method of substrate modification.

CONCLUSIONS

The scope of this work was to determine whether chain density (attributing to charge density) of imidazolium-containing PILs had any significant effect on antibacterial activity. This was tested by varying the UV initiator immobilization time, responsible for chain density, during substrate modification to form a poly(VImCl) PIL surface. These substrates were tested *against E. coli* and were found to have a substantial percent reduction in viable *E. coli* compared to the control substrate (52.3 ± 6.1 % vs. 0.0 ± 7.3 %, respectively). However, individual immobilization times had general trend of difference compared to other immobilization times in the amount of reduction. Future work should aim to achieve percent reduction values that are comparable to current

literature by using similar incubation conditions of *E. coli* during substrate testing and such as the volume of *E. coli* incubated and incubation time. This in hope would allow for any significant effects between each immobilization time to be differentiated and would give more comparable results to literature values.

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