Quaternized copolymers of 4-vinylpyridine and poly(ethylene glycol) methyl ether methacrylate are known to have antibacterial properties and have displayed biocompatibility in red blood cell hemolysis assays. The results from hemolysis assays have shown substantial promise, but the technique is rudimentary and only a first step toward the determination of biocompatibility. The present paper further explores the biocompatibility of these copolymers through comprehensive cell viability assays performed on Caco-2 human epithelial cells cultivated in vitro. We have shown that these copolymers are biocompatible at concentrations above their minimum bactericidal concentrations, leading to selectivity values that compare well with other microbicidal products.

Introduction

Polymeric microbicides are attractive due to their low cost, high reported efficacy, multiple applications, and the need to develop new tools to combat increasingly drug-resistant microbes.\(^1\)\(^–\)\(^7\) Two of the most widely studied classes of antimicrobial polymers are quaternary ammonium and pyridinium-based materials.\(^8\) One such material, poly(4-vinylpyridine) (PVP) quaternized by an alkyl bromide, is effective against a wide variety of microbes.\(^8\)\(^–\)\(^12\) However, this material lacks biocompatibility and is toxic to mammalian cells, even at low doses,\(^11\) thus limiting its applications. Previous work explored a novel method of improving biocompatibility through the copolymerization of 4-vinylpyridine (VP) with monomers that formed biocompatible homopolymers.\(^13,14\) This approach led to a surprising result: copolymers that included a small amount of the biocompatible monomer exhibited much stronger antibacterial properties than the quaternized PVP homopolymer. It is theorized for similar systems that this behavior is related to the amphiphatic balance\(^15,16\) or the spatial relationship of the cation to the alkyl chain.\(^17\) The following work showed that these copolymers have biocompatible potential, as they could also prevent the rupture of human red blood cells (RBC) in a hemolysis assay,\(^13\) and further work showed that the selectivity of these copolymers was comparable to that of other common microbicides.\(^18\)

As used in polymer microbicidal research, the term selectivity refers to the ratio of hemolytic concentration (HC\(_{50}\)) to the concentration of material that will lyse 50% of RBCs in an in vitro hemolysis assay, to the minimum bactericidal concentration (MBC), the lowest concentration that is able to kill a specified number of a species of bacteria (the MBC is occasionally referred to as the minimum lethal concentration, or MLC).\(^19\) Therefore, the ideal material will kill bacteria at concentrations orders of magnitude lower than it will lyse red blood cells, leading to a high selectivity. The MBC can be variable depending on the experimental system and test species chosen.\(^19,20\)

An alternative measure of bactericidal properties, the measurement of the material concentration needed to prevent the growth of new colonies, is known as the minimal inhibitory concentration, or MIC.\(^21,22\) The two measurements are related but distinct: MIC typically differs from the MBC by a factor of 2 to 4 (MIC typically being lower than MBC), depending on the material and testing procedure.\(^21,22\)

In vitro cell viability assays offer specific advantages over RBC hemolysis assays as a measure of biocompatibility. RBCs are frequently the first site of interaction between a biomaterial and bodily humors, especially for antibacterial materials with applications in burn and wound dressings. The RBC hemolysis assay requires minimal labor and no continuing maintenance of cell cultures. However, erythrocytes lack a nucleus, the ability to reproduce, and the ability to synthesize protein. Alternatively, in vitro cell cultures provide living, metabolizing cells undertaking a wide array of cellular functions. In hemolysis testing, erythrocytes are removed from the protective plasma proteins and bodily humors that mediate the interactions of RBCs.\(^23\) This provides the potential for misleading results due to the artificially clean (of protein) environment needed for the assay. In vitro cell viability testing directly exposes living cells to a material, providing for an alternative and in some ways advantageous method of exploring biocompatibility. Despite the value of cell viability testing on living cell samples, little to no research has examined the biocompatibility of polymer microbicides beyond hemolysis testing. This is perhaps due to the increased labor and sterile lab technique needed to successfully complete in vitro assays. The present article seeks to close this knowledge gap by reporting the results of cell viability assays performed on human intestinal epithelial cells cultivated in vitro.

Experimental Section

Polymer Synthesis and Characterization. The polymers and their synthesis are identical to those prepared in a previous publication,\(^18\) but a brief summary is given here. VP and poly(ethylene glycol) methyl ether methacrylate of molecular weight (\(M_n\) \(\sim\)1100 g/mol (PEGMA 1100) were synthesized through thermally initiated free radical polym-
Polymerization in chloroform using 2,2′-azobisisobutyronitrile (AIBN) as initiator, as shown in Figure 1 (all purchased from Sigma-Aldrich, St. Louis, MO). Following polymerization, precipitation, and characterization by 1H nuclear magnetic resonance spectroscopy (NMR),13,14 the polymers were redissolved in chloroform with 1-bromohexane, heated, precipitated, and dried forming poly(vinylpyridine-co-poly(ethylene glycol) methacrylate 1100)-hexylbromide, abbreviated P(VP-co-PEGMA 1100)-HB. Homopolymers of PEGMA 1100, poly(poly(ethylene glycol) methyl ether methacrylate) (PPEGMA 1100), were also synthesized through this method. Composition and quaternization were confirmed by 1H NMR,14 and gel permeation chromatography results indicated $M_n \sim 15\,000$ g/mol for all polymers. Copolymers were synthesized with monomer compositions of 0 mol % VP (i.e., the homopolymer of PPEGMA 1100), 10 mol % (i.e., 90 mol % PEGMA 1100), 50 mol %, 75 mol %, and 90 mol % VP to represent a broad spectrum of copolymer compositions (all future references to the percentage of VP in the polymers will refer to the molecular composition). Copolymers of greater than 90% VP as well as the quaternized homopolymer PVP-HB could not be studied in the cell viability assays due to their insolubility in aqueous solutions.

Cell Culture. We chose the human intestinal epithelial cell line Caco-2 (colorectal adenocarcinoma, ATCC HTB-37, Manassas, VA) because of its relevance as a human epithelial cell model system and its widespread use in pharmaceutical in vitro drug absorption assays24,25 and biocompatibility assays.24,26,27 The cells were grown according to ATCC’s recommended protocol using Eagle’s Minimum Essential Medium supplemented with 20% fetal bovine serum (ATCC). Cells were grown in 25 cm² or 75 cm² cell culture flasks (BD Biosciences, San Jose, CA) in a 5% carbon dioxide environment at 37 °C and 95% relative humidity. Flasks were split approximately every 96 h when they had reached 90% confluency with a 0.25% trypsin and 0.04% EDTA solution (Invitrogen, Carlsbad, CA). Once the cells had recovered from freezing, exhibited a consistent viability of 97% or higher through

**Figure 1.** Synthesis scheme and structure of quaternized P(VP-co-PEGMA 1100)-HB. Copolymers were synthesized through thermally initiated free radical polymerization in chloroform with AIBN as initiator, then quaternized with 1-bromohexane.

**Figure 2.** Fluorescence micrograph of Caco-2 cells after a 2 h exposure to P(VP-co-PEGMA 1100)-HB 90% VP copolymer at 0.1 mg/mL. Image is a composite of calcein AM (green) and EthD-1 (red) signal, which are viewed at separate emission wavelengths. Green cells represent cells that uptake and metabolize the calcein AM and are living cells. Red areas are the nuclei of dead or dying cells with disrupted membranes.

**Figure 3.** Relative counts of live and dead cells stained by LIVE and DEAD assay dyes for the copolymers of P(VP-co-PEGMA 1100)-HB. The 0% VP sample refers to the homopolymer of PPEGMA 1100. All polymer percentages refer to molecular composition. The starred (*) samples are those having a statistical significance of $p > 0.05$ (Student’s $t$ test) corresponding to the positive control.

**Figure 4.** Relative counts of live and dead cells stained by LIVE and DEAD assay dyes for the 75% VP copolymer of P(VP-co-PEGMA 1100)-HB. All polymer percentages refer to molecular composition. The starred (*) samples are those having a statistical significance of $p > 0.05$ (Student’s $t$ test) corresponding to the positive control.
Trypan Blue dye testing (Sigma-Aldrich), and had entered the growth phase, they were seeded into 12-well cell culture plates (BD Biosciences) at a concentration of 250,000 cells/well and a working volume of 1 mL. Assays were performed in the individual wells of the cell culture plates. Assays were performed in triplicate once the cells achieved greater than 90% confluency in each well. We dissolved our polymers in the culture media, heated the solutions to 37 °C, and incubated the confluent cells in these solutions for 2 h before application of the testing assays; this time period was chosen as it provided for substantial exposure and polymer contact without introducing the complicating effects of cellular growth in the samples (the doubling time of the cells is estimated to be 92 h). One set of cells was not exposed to any of the testing agents as a positive control, and one set of wells was fixed by exposure to 70% methanol (Sigma-Aldrich) for 30 min as a negative control.

Cell Viability. To measure the viability of our cells after exposure to the polymers we chose to use the LIVE/DEAD Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen), one of the most common assays for the determination of cell viability or cytotoxicity. The LIVE/DEAD assay kit uses two fluorescent dyes: a green calcein AM dye that is cleaved by ubiquitous intracellular esterase activity after being taken up by living cells, and an ethidium bromide homodimer-1 (EthD-1) dye that binds to nucleic acids but is unable to penetrate intact, healthy cell membranes. Our dyes were diluted in phosphate buffered saline (ATCC) to the manufacturer's recommended concentrations (2 μM calcein AM and 4 μM EthD-1) and incubated for 45 min prior to viewing by fluorescence microscopy at 100× on a Nikon Diaphot optical microscope (Tokyo, Japan) equipped with a QImaging Retiga 1300R camera (Surrey, BC). The calcein AM and EthD-1 images were taken separately using two sets of filters. The calcein AM was excited at 500 nm and fluorescence was observed at 520 nm. The EthD-1 was excited at 530 nm, and fluorescence was observed at 620 nm. Five randomly selected positions in each well were chosen for image capture and analysis. Exact cell counts were completed using the particle analysis function of Scion Image 4.02 (Frederick, MD). The positive control was determined by taking a sample of cells that were exposed to fresh media with no dissolved polymer during the incubation period. The negative control was determined by the results of exposing cells to 70% methanol for 30 min. The viability was determined by measuring the number of cells stained by the LIVE dye divided by the total number of cells dyed by either the LIVE or DEAD dyes for the sample. It should be noted that, although we have referred to our tests as measurements of cell viability, the use of positive and negative controls means that we could alternatively consider them as measurements of cytotoxicity. With this testing approach, there is a possibility for the undercounting of the dead cells due to cellular delamination from the surface of the culture dish (our copolymers are not known to have any fixative properties); however, a comparative analysis of the absolute living cell counts of the sample polymers versus the positive control sample enables us to consider the results independent of cellular delamination.

We performed the LIVE/DEAD assays on the positive and negative controls, the homopolymer PEGMA 1100, and each of the copolymers studied. We performed a serial dilution of each polymer applied to the cells, beginning at a concentration of 10 mg/mL or the limit of solubility in the media (see Table 1), and if biocompatible properties were not found, the polymer was diluted by an order of magnitude and tested again, until biocompatibility was obtained. It was observed that the solubility of the polymers in the media is lower in some cases than the solubility in water; given the additional salts and proteins in the cell culture media, this is not a surprising result. In testing, a polymer that displayed a percentage of viable cells with a statistical significance of \( p > 0.05 \) (Student’s \( t \) test) corresponding to the positive control was considered biocompatible, and will be termed a biocompatible result. This standard differs from that used in some RBC hemolysis tests, which measure the concentration of material that will lyse 50% of the cells, frequently referred to as HC_{50}. For our cell viability assays, we chose to measure a higher standard, the concentration at which the polymer is completely harmless over the 2 h exposure period (i.e., it shows no difference in cellular viability from the positive control). This standard is analogous to drug testing, where test subjects are statistically compared to the results of positive and/or negative controls. The biocompatible concentrations we have listed would be greater if we instead used as the standard the polymer concentration cell which lysed 50% or fewer of the cells, as is done in HC_{50} assays; however, in the projected applications for polymer microbicides like this one, a material which lyses 50% of human cells will not be helpful, and thus, we have decided to employ a more realistic standard.

As another measure of cell viability and toxicity, we examined the effect of the polymer on metabolic function using an “MTT” assay (ATCC), a reliable and widely accepted method of measuring cellular proliferation. The MTT assays consist of a yellow tetrazolium ((3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) dye that is cleaved by mitochondrial dehydrogenase enzymes to form purple, providing a measurement of mitochondrial (and therefore cellular) viability. This provides an alternative measurement of viability from the LIVE/DEAD assay, which primarily reports membrane integrity. The molecule created by the cleaving process of the MTT assay, formazan, absorbs light at 570 nm, thus enabling cell viability measurement through ultraviolet-visible (UV–vis) spectrophotometry. A sample with high cellular viability will absorb a high percentage of the light at 570 nm, while a sample with low or no viability will allow most light at 570 nm to be transmitted. As per the manufacturer’s recommended procedure, 100 μL of MTT dye was added to each well, allowed to incubate for 2 h, rinsed with 1 mL of a formazan-solubilizing detergent, and allowed to sit at room temperature for an additional 2 h before analysis in a Genesys 10b tunable spectrophotometer (Agilent, Santa Clara, Calif.). The zero absorbance point was calibrated to a sample of deionized water. The MTT assay was completed on both controls and the samples that were found biocompatible in the LIVE/DEAD assay as a confirmation of biocompatible behavior. The results from this absorbance assay are comparative: a polymer that displayed a percentage of viable cells with a statistical significance of \( p > 0.05 \) (Student’s \( t \) test) corresponding to the positive control was considered biocompatible, and will be termed a biocompatible result.

Results and Discussion

Results of the LIVE/DEAD Assay. We were able to observe the fluorescence of living and dead cells clearly using the aforementioned assay kit and fluorescence microscopy (see Figure 2). As expected, the living cells appear as diffuse green splotches, as the dye is taken into the entire cell membrane. Also, as expected, the dead cells appear as sharp red points, as this dye binds itself with the nuclei of ruptured cells. The results

![Figure 5. Relative counts of live and dead cells stained by LIVE and DEAD assay dyes for the 90% VP copolymers of P(VP-co-PEGMA 1100)-HB. All polymer percentages refer to molecular composition. The starred (*) samples are those having a statistical significance of \( p > 0.05 \) (Student’s \( t \) test) in correspondence with the positive control.](image-url)
are compared with a positive control that was determined by taking a sample of cells that were not exposed to any polymer and a negative control determined by exposing cells to methanol. The results of the LIVE/DEAD assay for all tested polymers and concentrations are displayed in Figures 3–5, averaged over the results of the triplicate experiments (error bars represent the standard deviation). The positive controls consistently reported viability above 98.6%, and the negative controls were consistently below 0.01% viability, as expected for healthy cells and cells exposed to methanol, respectively. The full results for the controls in each experiment, as well as the results in terms of absolute cell counts, are provided in the Supporting Information.

We observed biocompatible results for all materials, but at different concentrations of polymer exposure. The homopolymer PPEGMA 1100 and the copolymer containing 10% VP resulted in no detectable cell death (a biocompatible result) at the highest concentration tested (see Figure 3). The copolymers containing 50%, 75%, and 90% VP displayed less biocompatibility, presenting biocompatible results at 1 mg/mL for 50% VP, and at a much lower 10 µg/mL for 75% and 90% VP (see Figure 3 for 50% VP, Figure 4 for 75% VP, and Figure 5 for 90% VP). Some solutions showed results between the two extremes of 100% and 0% viability: specifically, 50% VP at 10 mg/mL (Figure 3) and 90% VP at 0.1 mg/mL (Figure 5). It is possible that the true limit of cell viability for these materials falls somewhere between the reported biocompatible concentration and the next highest tested concentration. Of particular interest is the biocompatible result for 50% VP at 1 mg/mL, which falls below the reported HC50 of 10 mg/mL in Allison et al.13 and the biocompatible result for 75% VP at 10 µg/mL, which is instead higher than the HC50 reported in Allison et al.13 Although these results differ from previous work, it is well-known in biocompatibility testing that different tests can produce widely differing results,29–31 and our cell viability assays match in principle the results from Allison et al.,13 providing additional support for the biocompatible nature of these copolymers.

Results of MTT Testing. The MTT assay to measure mitochondrial dehydrogenase activity was performed as a second measure of cell viability and function. The results of the MTT assay on the biocompatible copolymers, control samples, and the minimally biocompatible sample 90% VP at 0.1 mg/mL are provided. This test is entirely comparative, using the positive and negative controls to set the limits of viability and toxicity. Higher absorbance of the 570 nm light indicates increased metabolic activity within the well. The results, displayed in Figure 6, are plotted in units of the percentage of light absorbed. As expected, all of the materials with high cell viability (as determined from the LIVE/DEAD assay) show high absorbance at 570 nm and show a statistically significant correlation (p > 0.05) with the positive control. The negative control, in comparison, shows the low absorbance indicative of a lack of mitochondrial activity, as expected for cells killed and fixed by methanol. This assay therefore provides additional confirmation of the biocompatible nature of these polymers. One interesting result is the performance of P(VP-PPEGMA 1100)-HB 90% VP at 0.1 mg/mL, which falls only just outside the margin of error of the control sample but within the bounds of statistical significance, despite killing 45% of the cells as measured from the LIVE/DEAD assay. It is possible that the MTT assay shows less sensitivity due to the nature of the polymer interaction with the cells, e.g., that the polymers may be able to penetrate the cell membranes over the 2 h time frame, but are less adept at interrupting dehydrogenase function, or some other potential mitigating factor. This result does not diminish the value of the assay as a confirmation of the biocompatible results concurrently found in the LIVE/DEAD assay, but illustrates the challenges inherent in the determination of biocompatible properties.

Selectivity. Previous work by Allison et al.13 and Stratton et al.18 determined the HC50, MBC, and selectivity for some of these copolymers, which are listed in Table 2. We will refer to the selectivity as determined by comparing the HC50 and MBC as hemolytic selectivity. MBC was determined by the ability of the polymer to kill 10⁶ colonies of Escherichia coli O157:
Table 2. Comparison of Minimum Bactericidal Concentrations (MBCs), Hemolytic Concentrations (HC50), Cell Viability Concentrations (LC50), and Their Respective Selectivity Values for the Homopolymer PPEGMA 1100 and the Quaternized Copolymers of P(VP-co-PEGMA 1100)-HB

<table>
<thead>
<tr>
<th>Material</th>
<th>MBC (µg/mL)</th>
<th>HC50 (µg/mL)</th>
<th>Hemolytic selectivity</th>
<th>LC50 (µg/mL)</th>
<th>Viability assay selectivity</th>
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<tbody>
<tr>
<td>PEGMA 1100</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P(VP-co-PEGMA 1100)-HB 10% VP</td>
<td>ineffect</td>
<td>10 000</td>
<td>ineffect</td>
<td>1000</td>
<td>ineffect</td>
</tr>
<tr>
<td>P(VP-co-PEGMA 1100)-HB 50% VP</td>
<td>70</td>
<td>10 000</td>
<td>140</td>
<td>1000</td>
<td>14</td>
</tr>
<tr>
<td>P(VP-co-PEGMA 1100)-HB 75% VP</td>
<td>~40 (est.)</td>
<td>1</td>
<td>~0.025</td>
<td>10</td>
<td>~0.3</td>
</tr>
<tr>
<td>P(VP-co-PEGMA 1100)-HB 90% VP</td>
<td>5</td>
<td>&lt;1</td>
<td>~0.2</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

H7 as reported in previous results. The 75% VP copolymer was found to be effective against bacteria, but an MBC for this copolymer was not calculated; however, on the basis of previous results, it can be inferred that the MBC for the 75% VP copolymer is somewhere between that of the 50% VP and 90% VP copolymers. From the results of our cell viability assays, we can determine the selectivity of the copolymers by comparing the maximum concentration of polymer that does not lyse any cells (hereafter referred to as LC50) vs the MBC results as reported from earlier data, which we will refer to as “viability assay selectivity”. Table 2 contains the complete tabulation of these results. Although the viability assay selectivity of the copolymers may appear disappointing in comparison to those results derived by hemolysis testing, this is not surprising given the results of other publications in this area which have shown HC50 results almost an order of magnitude larger than LC50 results. Regardless, the selectivity of our polymers is still broadly comparable to others in the field of polymer microbicides, all of which were measured with RBC hemolysis testing. Our material may even have a higher selectivity than comparable materials, as other studies frequently measured MIC instead of MBC. It is also certainly possible that these comparable materials may also have lower selectivity values when compared to the results of an in vitro assay like ours. Further testing with additional assays and cell lines would provide greater support for the biocompatibility of these polymers, as different biocompatibility assays or cell lines can exhibit differing results.

Conclusions

We reported on the results of cell viability assays that were completed on previously developed copolymers of VP and PEGMA 1100. We found that the antibacterial copolymers showed promising results in biocompatibility and received reasonably consistent results between the two common cell viability assays. Future work could improve upon these experiments in a variety of ways. Additional cell viability assays and cell lines would provide further evidence for the biocompatibility of these copolymers, as well as the many other microbicidal polymers in development. In our experiments, we focused on two common assays that measure specific aspects of viability: the LIVE/DEAD assay, which reports on the permeability of the cell membrane, and the MTT assay, which reports on the vitality of mitochondrial function. These assays together do not report on all aspects of cellular function and metabolism, nor do they shed any valuable light on the method of action of quaternary cationic microbicides. Completing further assays would provide additional experimental evidence for or against biocompatibility, and could even provide information on their method of action. Our testing methodology made use of commonly available assays and materials to provide consistent results, but with limited throughput. A potential method for improving the throughput of the testing procedure through the use of multiwell plates with smaller wells (96-well plates are common) to decrease the labor involved in the production of cultures for sampling. However, in order to culture, feed and test, and analyze so many samples quickly, automated testing equipment would be highly desirable or perhaps necessary. The implementation of a robotic, automated procedure would enable the high-throughput testing of a larger number of materials, cell lines, and assays, increasing the potential support for the biocompatible nature of a chosen material.

In vitro cell viability assays provide a useful tool for predicting biocompatibility along with the more common RBC hemolysis assay. The RBC hemolysis assay requires less equipment and labor per sample, but is able to provide only one measurement of biocompatibility for one type of tissue. Given their contrasting advantages and disadvantages, the two should be used in concert to provide the greatest information possible on the biocompatibility of an analyzed material. Although the selectivity values gleaned from these experiments were not greater than those gained from hemolysis testing, their reasonably similar results show that the results of RBC hemolysis testing were not incidental, but rather indicative of a biocompatible nature of a chosen material. Further work on understanding the biocompatibility of polyquats and other antibacterial copolymers will shed more light on the complex relationships of these materials with living systems.

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Supporting Information Available. The exhaustive quantitative results of the LIVE/DEAD assay for each sample run. The material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes