Articles

Hemocompatibility of Hydrophilic Antimicrobial Copolymers of Alkylated 4-Vinylpyridine

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Quaternized poly(vinylpyridine) (PVP) is a polymer with inherent antimicrobial properties that is effective against Gram-positive bacteria, Gram-negative bacteria, viruses, and yeast cells. However, quaternized PVP has poor biocompatibility, which prevents its use in biomaterial applications. Copolymerization was examined as a method of modifying the structure to incorporate biocompatibility. Polyethyleneglycol methyl ether methacrylate (PEGMA) and hydroxyethyl methacrylate (HEMA) are polymers generally known to be biocompatible and thus were chosen as comonomers. Random copolymers of 4-vinylpyridine and PEGMA or HEMA were synthesized via free radical polymerization and quaternized with bromohexane. Copolymer biocompatibility was characterized by interaction with human red blood cells to analyze hemolysis. Hemolysis of human red blood cells was conducted on insoluble films and on water-soluble polymers in a serial dilution study. Hemolysis results demonstrated that blood compatibility does not depend on PEG chain length in PEGMA incorporated copolymers. Results indicate a critical weight ratio of PEGMA to VP in copolymers separating the no-hemolysis regime from 100% hemolysis.

Introduction

Products commonly marketed as antibacterial are effective because they are impregnated with agents such as triclosan or silver ion. These agents are limited by short effective lifetimes[1,2] due to leaching and bacterial resistance.[3,4] Materials that kill or prevent the growth of bacteria because of structure have potential in biomaterial applications where infections are common. This would overcome the need for labor-intensive antiseptic treatments and the short lifetimes of agents currently leached from "antibacterial" products.

Cationic polymers, such as poly(4-vinyl-*N*-alkylpyridinium bromide), have been proven effective at killing bacteria because of their structure.[2,5,6] Electrostatic attraction of the cation to the negatively charged bacteria membrane allows for disruption of the membrane by the quaternizing alkyl tail, which leads to cell death.[7] Recently, it was shown that alkylated pyridinium and ammonium polymers, covalently attached to glass slides, could create a surface that killed airborne and waterborne bacteria on contact.[8,9] A variety of drug-resistant pathogens, including methicillin- and penicillin-resistant bacteria,[8] are affected by poly(vinyl-*N*-hexylpyridinium bromide). These antibacterial moieties have also been attached to the surface of common woven textiles such as cotton, wool, nylon, and polyester.[8,9] However, materials with quaternary ammonium salt structures suffer from poor biocompatibility and irritation to mammalian cells,[10,11] such as skin, limiting their use in biomaterial applications.

Chemical structure and surface properties are two variables commonly examined to understand the biocompatibility of polymers.[12−14] Hydrophilic polymers are commonly found in biomaterial applications as they impede the hydrophobic−hydrophobic interactions necessary for large amounts of protein adsorption. Additionally, uncharged materials are preferred because they are not electrostatically attracted to cells and have little surfactant behavior and so do not lyse biological structures. Two examples of hydrophilic biopolymers are poly(ethylene glycol) (PEG) and poly(hydroxyethylmethacrylate) (PHEMA). PEG is a linear soluble polymer that exhibits low toxicity, is FDA approved for internal consumption, and is known to protect human red blood cells.[14,15] PHEMA is a hydrogel most notably use in soft contact lenses.

Studying host response to biomaterials in a blood-contacting environment is key to understanding hemocompatibility with respect to toxicity. The biomaterial interface contacts both cellular components of blood, namely red blood cells (RBCs). RBCs can undergo hemolysis by materials interactions, which releases free hemoglobin into the blood stream. Free hemoglobin released beyond a critical threshold can cause anemia and/or kidney failure. Mechanical stress, blood dilution, and interaction with foreign materials can all contribute to hemolysis and possibly to toxic effects, which is a first step necessary in understanding hemocompatibility by material−host interactions.

Previously, we reported that incorporation of hydrophilic functional groups with Q-PVP enhances bacterial killing efficency.[16] In an effort to design bactericidal materials with improved hemocompatibility, we synthesized copolymers of 4-vinylpyridine (4-VP) with monomers known to be strongly hydrophilic and biocompatible: hydroxyethylmethacrylate (HEMA) and polyethylene glycol methyl ether methacrylate

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(PEGMA).[17] The polymers were then analyzed for hemocompatibility through a human red blood cell lysis assay.

Materials and Methods

Material. 2,2′-Azobisisobutyronitrile (AIBN), 1-bromohexane, 4-vinylpyridine (VP), 2-hydroxyethyl methacrylate (HEMA), poly(ethylene glycol) methyl ether methacrylate (PEGMA) of Mn ~1100 g/mol, 475 g/mol and ~300 g/mol, respectively, Tris-buffered saline (Tris), methanol, chloroform, polyethylene glycol with a molecular weight of 900, and Triton-X 100 were purchased from Sigma-Aldrich (St. Louis, MO).

VP and HEMA were purified by vacuum trap-to-trap distillation. PEGMA was purified by column chromatography eluted in chloroform, with 70−240 μm silica mesh acting as the stationary phase. Tris-buffered saline powder was dissolved in deionized water as instructed. All other materials were used as received.

Copolymer Synthesis. The synthesis procedure is modified from Sellenet et al.[16] Copolymers were synthesized by free radical polymerization by using AIBN as the thermal initiator. Mole ratios of VP to HEMA and PEGMA were varied across the composition spectrum. A total of four composition sets were produced: VP/HEMA, VP/PEGMA-300, VP/PEGMA-475, and VP/PEGMA-1100. VP/comonomer ratios synthesized for HEMA polymers comprised 9/91, 12/88, 27/63, 54/46, 75/25, 88/12, 94/6, and 98/2. VP/PEGMA-300 comonomer ratios were synthesized at ratios of 3/97, 8/92, 30/70, 38/62, 45/55, 52/48, 75/25, 90/10, 95/5, and 99/1. VP/PEGMA-475 comonomer ratios were synthesized at ratios of 2/98, 7/93, 12/88, 30/70, 35/65, 40/60, 52/48, 77/23, 90/10, 95/5, and 99/1. VP/PEGMA-1100 comonomer ratios were synthesized at ratios of 1/99, 5/95, 12/88, 25/75, 35/65, 60/40, 64/36, 69/31, 75/25, 90/10, 95/5, and 99/1. Total monomer volume was held constant to 3.5 mL. AIBN was held at a constant mass of 0.5 g for all reactions. PEGMA reactions were conducted by using chloroform as the solvent, while HEMA reactions were performed in methanol. Reactions were conducted in 20 mL scintillation vials with a Teflon stir bar. Monomers and AIBN were mixed and dissolved in solvent and vials were evacuated and saturated with nitrogen three times to ensure that no oxygen was contained in each vial before being sealed. Vials were stirred and heated in an oil bath at 70 °C for 48 h. Copolymers were precipitated in hexane three times and dried under vacuum for 12 h at 90 °C.

Proton NMR and GPC were performed to determine composition and molecular weight. Polymers were slightly deficient in acrylate monomer, with r_{vp}/r_{CM} values (Kelen−Tudos method) of 0.83/0.65, 0.87/0.63, 0.99/0.78, and 1.13/0.80 for HEMA, PEGMA-300, PEGMA-475, and PEGMA-1100, respectively. R_1R_2 values increased from 0.54 to 0.9 from HEMA to PEGMA-1100, indicating a generally random structure. As resultant polymers differ from feed ratios by a small margin, polymers will be referred to by their feed compositions to maintain uniformity. GPC was not performed on HEMA polymers due to insolubility in the eluent (THF), however, PEGMA series polymers that were soluble were analyzed. PEGMA-300 polymers showed molecular M_n decreasing from 35 000 to 10 000 g/mol as VP content was increased with PDIs constant at ~1.8. PEGMA-475 polymers showed constant M_n ~20 000 g/mol and PDIs ~1.6. PEGMA-1100 polymers showed constant M_n ~15 000 g/mol with PDIs ~1.3.

Quaternization. Copolymers were redissolved in their respective solvent and 1-bromohexane was added at a molar ratio of 3:1 to quaternize the pyridine ring. Each reaction vessel was frozen, pumped, thawed, and saturated with nitrogen, then resealed and stirred at 70 °C for 72 h. Copolymers were then dried at 90 °C for 12 h. The final copolymer structures of quaternized of P(VP-*co*-PEGMA) and quaternized P(VP-*co*-HEMA) can be seen in Figure 1.

FTIR was used to follow the quaternization[18] with subsequent final characterization with ^{1}H NMR from which copolymer compositional ratios were calculated. Measured copolymer compositions were used for analysis of hemolysis results rather than feed ratios. Molecular weight distributions of the copolymers were obtained by GPC and molecular weight results ranged between 20 000−40 000 g/mol.

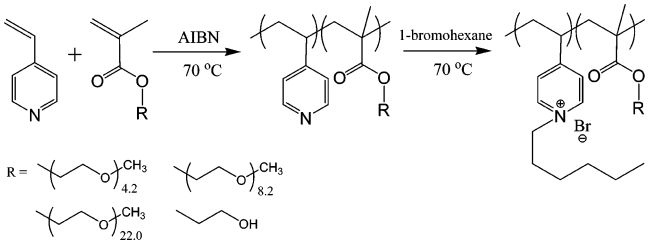

Figure 1. Synthetic scheme of quaternized P(VP-*co*-PEGMA) M_n = 300 g/mol (n = 4.2), 475 g/mol (n-8.2), 1,100 g/mol (n = 22.0) and quaternized P(VP-*co*-HEMA).

Hemocompatibility. The procedure for hemolysis was modified from Ilker et al.[19] Fresh human blood, 30 μL, was suspended in 10 mL of Tris-buffered saline (Tris) and centrifuged at 1500 rpm for 5 min to separate, rinse, and retain red blood cells (RBCs). Supernatant was removed and RBCs were resuspended and rinsed 3 times. Water soluble polymers were dissolved in Tris to an initial concentration of 20 mg/mL. Polymer solutions, 0.75 mL, and RBC-Tris solution, 0.75 mL, were mixed in centrifuge tubes to a final polymer concentration of 10 mg/mL. Tubes were then tumbled in a rotisserie hybridization oven at 37 °C for 30 min. Samples were then centrifuged at 3000 rpm for 10 min. Free hemoglobin in the supernatant was measured with a DU800 UV−vis spectrophotometer at 414 nm. Triton-X, a surfactant known to lyse RBCs, was the positive control, and samples were normalized to 10 mg/mL (1 wt %) Triton-X as 100% hemolysis. RBC-Tris solution and PEG (M_n 900) in Tris at 10 mg/mL were the negative controls. A serial dilution of each sample was performed until no hemolysis was observed.

Aqueous hemolysis tests were modified to characterize non-water-soluble copolymers. Thin-films of non-water-soluble copolymers were formed by slowly evaporating solutions of HEMA and insoluble PEGMA copolymers in centrifuge tubes at room temperature. RBCs were rinsed as previously stated. Each centrifuge tube was exposed to a mixture of 0.75 mL of blood solution and 0.75 mL of Tris saline. Free hemoglobin measurements were performed in the same manner as the soluble samples.

In each case, four different tests were performed. Polymers solutions or films were prepared separately for analysis. Different blood samples were taken and prepared in each case along with a separate Triton-X control. Values are the average of these four tests, with error bars to 1 standard deviation.

Results

Up to a mole ratio of 90/10 (VP/PEGMA), PEGMA-based copolymers are water-soluble. Copolymers containing at least 90 mol % VP and all HEMA copolymers were insoluble. Those found to be soluble were then measured for interaction with red blood cells in solution, while those that were insoluble were analyzed for hemolysis as films.

Hemolysis results of RBCs interacting with water-soluble PEGMA copolymers are presented in Figure 2. The results show that, as VP content of the copolymers increases, blood response to the polymers switches from almost no hemolysis to 100% hemolysis at a critical molar content. Hemolysis occurred at 30, 35, and 64 mol % VP content for PEGMA 300, 475, and 1100 copolymers, respectively. This indicates that hemolysis was reduced (with greater bactericidal content) as longer PEG chain comonomers were incorporated on a mole basis.

Hemolysis data was further analyzed as a function of wt % PEG and is presented in Figure 3. The hemolysis step function for all copolymer series shifted to single value. Hemolysis data for PEGMA-based copolymers collapsed into a single-step

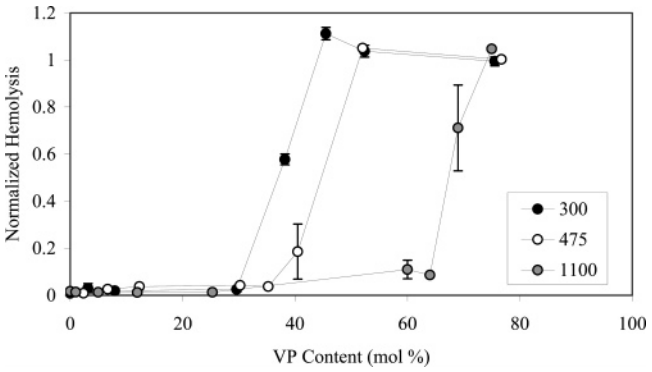

Figure 2. Human red blood cell (RBC) hemolysis of water soluble P(VP-*co*-PEGMA) at a concentration of 10 mg/mL as a function of VP mol %. Values are relative to hemolysis by 1% Triton-X solution.

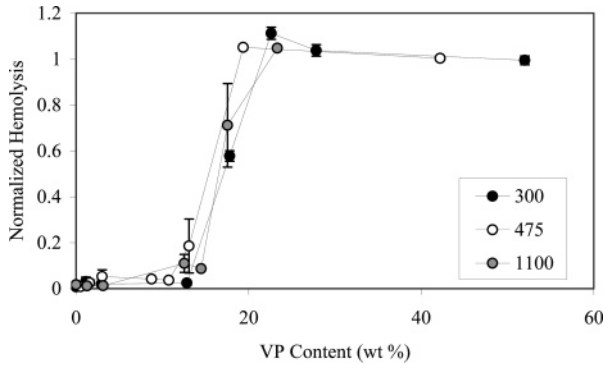

Figure 3. Human red blood cell (RBC) hemolysis of water soluble P(VP-*co*-PEGMA) at a concentration of 10 mg/mL as a function of VP content wt %. Values are relative to hemolysis by 1% Triton-X solution.

function curve with hemolysis occurring at 12−15 total wt % of VP and above in a 1% solution. That hemolysis occurs at approximately the same value reveals that blood compatibility of PEGMA copolymers is only a function of total PEG in the system and is not dependent upon the length of the PEG chain in each copolymer.

A serial dilution of each soluble copolymer solution was performed starting at 10 mg/mL until no hemolysis was observed and is presented in Figure 4. On the basis of results in Figure 3, these plots are shown as a function of wt % VP. As concentration is decreased, there a shift in the hemolysis step to greater VP contents, although the shift is small, on the order of 5−10 wt %. This indicates that VP content is important to activity against RBCs while solution concentration plays a minor role (note: due to a lack of data points in the transition region, the PEGMA 475 copolymer seems to have a large shift, but this effect may be illusory).

PEGMA-based copolymers with at least 90 mol % VP, which were previously shown to be water insoluble, were found to all cause 100% hemolysis by interaction of films with RBCs. These results are not shown, but this indicates that the antibacterial unit causes hemolysis by interaction of films with RBCs, similar to that seen in solutions.

Hemolysis of the HEMA series of copolymers interacting with RBC is presented in Figure 5. The step function of hemolysis occurred at 8−10 mol % VP in the copolymer. PHEMA is generally considered biocompatible and has been used in non-blood-contacting applications, although unlike PEG, PHEMA has not been shown to protect RBCs. Therefore, hemolysis occurring at such low VP content was not surprising.

The 9% and 12% VP content specimens have much larger statistical error than the other points. This error is due to the fact that, on different independent measurements, these specimens would show near-zero or near-unity hemolytic behaviors. For the 9% material, values of 0.10, 0.18, 1.07, and 0.06 were obtained in consecutive runs, and for the 12% material, values of 0.16, 1.00, 1.11, and 0.10 were obtained in the same respective consecutive runs. We are uncertain as to the origin of this effect; however, it seems confined to near the step from zero to unity hemolysis and to the HEMA copolymers (i.e., these two points).

Discussion

An explanation of the results derives from the effect of two functional groups on our polymers that have distinctly different properties, yet whose interplay dictates the RBC response: the alkyl chain and the hydrophilic group, although for brevity we will focus on the PEG-based polymers. It is generally believed that the alkyl chain of bromohexane-quaternized VP acts as a bactericide by lysing these cells in a manner similar to the action of surfactants. However, these surfactants also lyse RBCs, and we believe that a similar action of the alkyl chain of the polymer disrupts the membrane of RBCs causing them to rupture.

In opposition to the lysing effects of the alkyl chain, PEG is a water-soluble polymer that has been shown to reduce mechanical hemolysis of RBCs. For this reason, PEG of molecular weight 900 g/mol was analyzed as a negative control and is presented along with RBC in Tris and soluble P(VP-*co*-PEGMA) in Figure 6. Within standard deviation, the two values are the same, however, the test is not optimized for such low levels of hemolysis and so the protective effect may not show. In many PEGMA copolymers, hemolysis is significantly lower than RBCs interacting with Tris, showing that our materials have a PEG-like protective behavior with the PEG functional group, enhancing hemocompatibility.

The explanation of the hemoprotection of PEGylated materials lies with how the experiment used in this research exposes RBCs to a worst case scenario in rinsing blood to remove plasma proteins. Plasma proteins act as the natural protective agent for RBCs[20] by adsorbing to the cell membrane and preventing contact with cells, proteins, and other foreign bodies as well as shear stresses from fluid flow. By removing these proteins, RBCs are more fragile and are susceptible to shear stress and lytic agents. PEG acts as a protective agent in a similar manner to plasma proteins by interfering with foreign body contact. There has also been evidence that PEG weakly adsorbs to the cell membrane through hydrogen bonds of the hydroxyl group to enhance the PEG concentration at the cell surface, enhancing the protective effect.[14] Therefore, that our PEG-based polymers show a protective effect similar to PEG alone is not surprising. What is curious, however, is that certain PEGMA copolymers exhibit significantly lower hemolysis than the PEG negative control. The reason is unclear but may be a molecular weight effect, as the polymer is much larger than the PEG alone. Another possibility is that the cationic nitrogen on the polymers enhance adsorption to the cell wall, thereby increasing the local concentration of protective polymer.

When analyzing the serial dilution data, the extreme activity of the polymers becomes obvious. All materials that caused hemolysis were active down to 0.001 mg/mL level, while the minimum concentration where Triton-X 100 was still active was 0.01 mg/mL. The activity is possibly due to the larger molecular weight and resultant multivalency of the copolymer structure. An additional possibility is the greater attraction that the cationic structure of the polymer has for the cells over the neutral

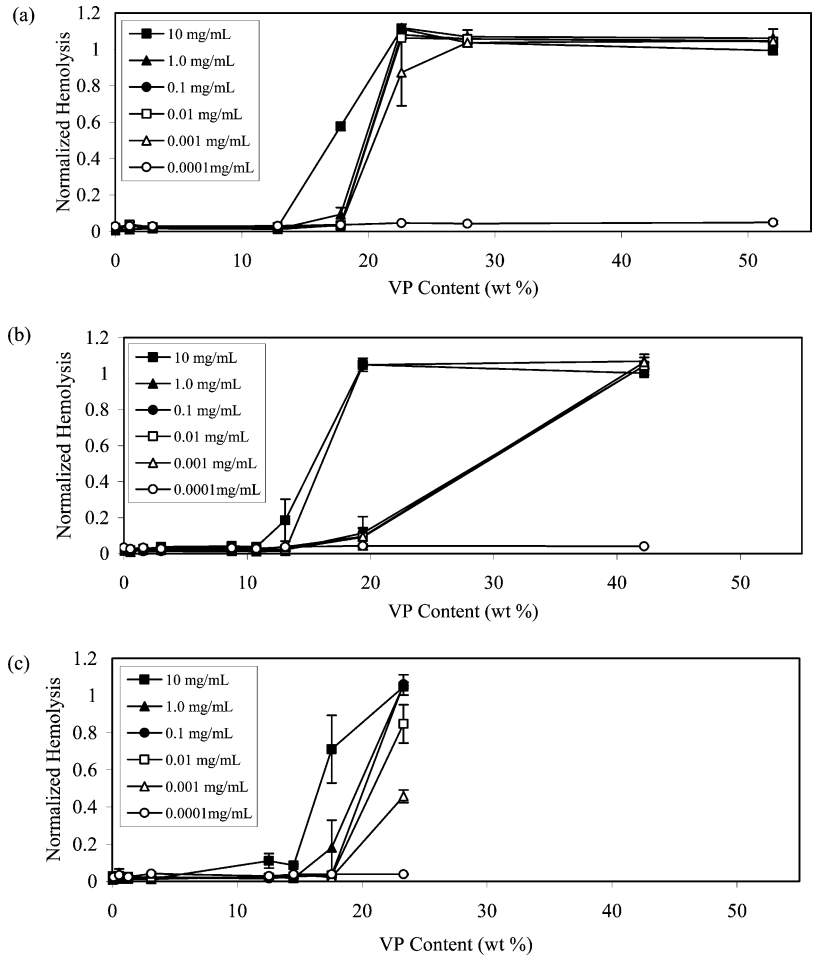

Figure 4. Human red blood cell (RBC) hemolysis of P(VP-*co*-PEGMA) (a)300 (b)475 (c)1100 solutions at concentrations of −■− 10 mg/mL, −▲− 1.0 mg/mL, −●− 0.1 mg/mL, −□− 0.01 mg/mL, −△− 0.001 mg/mL, and −○− 0.0001 mg/mL as a function of VP content (wt %). Values are normalized to hemolysis by 1% Triton-X solution.

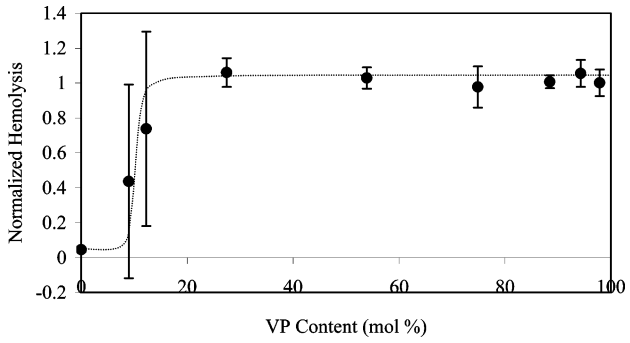

Figure 5. Human red blood cell (RBC) hemolysis of P(VP-*co*-HEMA) as a function of VP content wt %. Values are relative to hemolysis by 1% Triton-X solution. (line added to guide the eye)

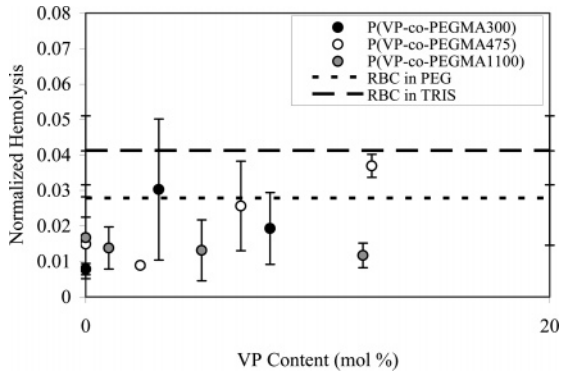

Figure 6. Magnified view of low VP content region of P(VP-*co*-PEGMA) hemolysis data compared to controls: PEG900 and Tris at a concentration of 10 mg/mL. Values are relative to hemolysis by 1% Triton-X solution.

structure of the Triton-X, thus causing a lower effective dose needed to lyse the RBCs. A surprising result is that hemolysis behavior did not show a high degree of dose dependence. The results show that, for polymers able to cause hemolysis, there is a critical concentration beyond which all cells were lysed and below which no cells were lysed, although there is a small shift in the monomer content necessary for hemolysis.

Looking at the data in aggregate, there is no obvious effect of longer PEG chains on the hemolytic behavior of the materials. In many ways, this result is surprising as previous research has shown that longer PEG chains are more effective at preventing protein adsorption on surfaces and stabilizing RBCs in solution.[14] However, this effect is not tied to the molecular structure but to the ability of longer PEG chains to keep interacting bodies further apart. For this reason, others have shown that bottle-brush structures where PEG is the side chain can work equally well.[21,22] Our analysis is that the copolymers here act in a similar way to the bottle-brush structures. Because the longer chain PEG monomers have a lower molar monomer content in the copolymer, the net effect of keeping RBCs away and shielding the lytic moieties on the polymer are the same.

Summary

In an effort to prepare biocompatible bactericidal materials, 4-vinylpyridine was copolymerized with polyethylene glycol methyl ether methacrylate or hydroxyethyl methacrylate by free radical polymerization and quaternized with bromohexane.

Blood compatibility was tested to understand interactions of synthesized copolymers with red blood cells. Results showed that addition of PEG to the polymers prevented hemolysis below a critical VP content of 12−15 wt % while insoluble HEMA-based films showed the hemolysis limit at 8−10 mol %. A serial dilution was performed on soluble polymers until no hemolysis occurred for any composition, which was achieved at 0.0001 mg/mL, an order of magnitude lower than the Triton-X positive control, indicating that a multivalent polymeric structure enhances hemolytic activity. Study of hemolysis also showed that red blood cell compatibility was not enhanced by longer PEG chains and that total PEG by weight was the critical design parameter for determination of hemolysis, although a minor effect of polymer concentration was also observed. Results also show an apparent protective effect of certain nonhemolytic polymers on the RBCs above that of PEG alone.

Comparison to results from previous work[16] on the efficacy of such hydrophilized antimicrobial polymers is difficult owing to the different testing formats of the materials (note: previous results did not include PEGMA-475 copolymers). For the data presented here, the measurements were performed in solution, allowing serial dilution to determine the minimum active concentration. The previous bacterial testing was performed on a coated surface where dissolution kinetics and wettability were dominant factors in the behavior. This difference in testing format prevents much comparison; however, generalizations can be made. The previous efficacy results showed that small additions (5−10 mol %) of hydrophilic monomer greatly increased the efficacy of the materials with this behavior attenuated as comonomer content is increased. Looking to the results of the current study, data show that formulations that were better than polymers alone were also are highly hemolytic. This result may imply commonality of action and may limit applicability in blood contact situations unless further molecular design is done.

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References and Notes

(1) Cen, L.; Neoh, K. G.; Kang, E. T. *J. Biomed. Mater. Res., Part A* **2004**, *71*, 70−80.
(2) Nohr, R. S.; Macdonald, J. G. *J. Biomater. Sci., Polym. Ed.* **1994**, *5*, 607−619.
(3) Heath, R. J.; Rubin, J. R.; Holland, D. R.; Zhang, E. L.; Snow, M. E.; Rock, C. O. *J. Biol. Chem.* **1999**, *274*, 11110−11114.
(4) Chuanchuen, R.; Beinlich, K.; Hoang, T. T.; Becher, A.; Karkhoff-Schweizer, R. R.; Schweizer, H. P. *Antimicrob. Agents Chemother.* **2001**, *45*, 428−432.
(5) Isquith, A. J.; Abbott, E. A.; Walters, P. A. *Appl. Microbiol.* **1972**, *24*, 859−863.
(6) Senuma, M.; Tashiro, T.; Iwakura, M.; Kaeriyama, K.; Shimura, Y. *J. Appl. Polym. Sci.* **1989**, *37*, 2837−2843.
(7) Kawabata, N.; Nishiguchi, M. *Appl. Environ. Microbiol.* **1988**, *54*, 2532−2535.
(8) Lin, J.; Qiu, S.; Lewis, K.; Klibanov, A. M. *Biotechnol. Bioeng.* **2003**, *83*, 168−172.
(9) Abel, T.; Cohen, J. I.; Engel, R.; Filshtinskaya, M.; Melkonian, A.; Melkonian, K. *Carbohydr. Res.* **2002**, *337*, 2495−2499.
(10) *4-Vinylpyridine*; MSDS no. V3204; Sigma-Aldrich: St. Louis, MO, 2006.
(11) *Octyltrimethylammonium Chloride*; MSDA no. 75094; Sigma-Aldrich: St. Louis, MO, 2006.
(12) Sevastianov, V. I.; Drushlyak, I. V.; Eberhart, R. C.; Kim, S. W. *Macromol. Symp.* **1996**, *103*, 1−4.
(13) Wang, Y. X.; Robertson, J. L.; Spillman, W. B.; Claus, R. O. *Pharm. Res.* **2004**, *21*, 1362−1373.
(14) Kameneva, M. V.; Repko, B. M.; Krasik, E. F.; Perricelli, B. C.; Borovetz, H. S. *ASAIO J.* **2003**, *49*, 537−542.
(15) Fu, J.; Fiegel, J.; Krauland, E.; Hanes, J. *Biomaterials* **2002**, *23*, 4425−4433.
(16) Sellenet, P. H.; Allison, B.; Applegate, B. M.; Youngblood, J. P. *Biomacromolecules* **2007**, *8*, 19−23.
(17) Abraham, S.; Brahim, S.; Guiseppi Elie, A. In *Proceedings of the 26th Annual International Conference of the IEEE*; San Francisco, CA, 2004.
(18) Liu, C. H.; Xiao, C. B. *J. Appl. Polym. Sci.* **2004**, *92*, 559−566.
(19) Ilker, M. F.; Nusslein, K.; Tew, G. N.; Coughlin, E. B. *J. Am. Chem. Soc.* **2004**, *126*, 15870−15875.
(20) Kameneva, M. V.; Antaki, J. F.; Yeleswarapu, K. K.; Watach, M. J.; Griffith, B. P.; Borovetz, H. S. *ASAIO J.* **1997**, *43*, M571−M575.
(21) Andruzzi, L.; Senaratne, W.; Hexemer, A.; Sheets, E. D.; Ilic, B.; Kramer, E. J.; Baird, B.; Ober, C. K. *Langmuir* **2005**, *21*, 2495−2504.
(22) Youngblood, J. P.; Andruzzi, L.; Ober, C. K.; Hexemer, A.; Kramer, E. J.; Callow, J. A.; Finlay, J. A.; Callow, M. E. *Biofouling* **2003**, *19*, 91−98.

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