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Antimicrobial Polymethacrylates Synthesized as Mimics of Tryptophan-Rich Cationic Peptides

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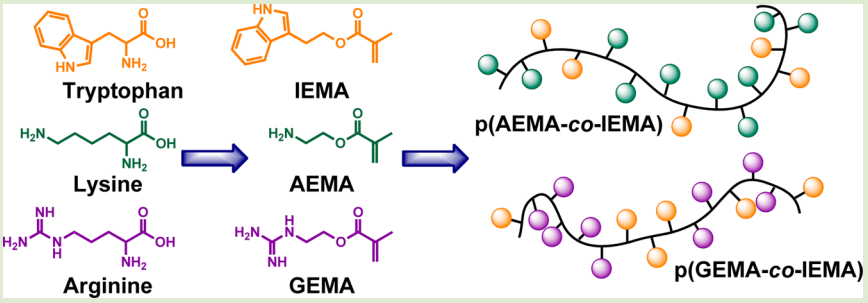
Supporting Information



ABSTRACT: This study describes a facile and high yielding route to two series of polymethacrylates inspired by the naturally occurring, tryptophan-rich cationic antimicrobial polymers. Appropriate optimization of indole content within each gave rise to polymers with high potency against *Staphylococcus epidermidis* (e.g., PGI-3 minimum inhibitory concentration (MIC) = 12 μg/mL) and the methicillin-resistant strain of *Staphylococcus aureus* (e.g., PGI-3 MIC = 47 μg/mL) with minimal toxicity toward human red blood cells. Future work will be directed toward understanding the cooperative roles that the cationic and indole pendant groups have for the mechanism of these polymers.

Naturally occurring antimicrobial peptides (AMPs) form an integral part of an organism's host defense system. Many of these peptides have been shown to possess broad spectrum antimicrobial activity concordant with low mammalian cell toxicity and a low susceptibility to the development of bacterial resistance.[1] The reduced prevalence of resistance is derived from the fact that AMPs are thought to bind to and disrupt membrane function rather than through specific receptor−protein interactions as with classical antibiotics. For these reasons, AMPs and their synthetic antimicrobial peptide mimics[2−6] have attracted increasing interest as a new hope for the development of novel agents to fight the increasing rate of antibiotic resistance seen in our healthcare system. There are, however, associated drawbacks to the use of peptides as therapeutic agents as they typically have limited pharmacokinetic and chemical stability and can be costly to produce on a large scale.

To overcome this, the focus has now switched to methods that allow chemists to synthetically capture the essential characteristics of AMPs within a synthetic polymer construct. This has spurred the development of a huge variety of synthetic AMP mimics based around various polymer backbones including polyvinylpyridines,[7] polyanilines,[8] polycarbodiimides,[9] polynorbornenes,[10] nylon-3 copolymers,[11] and polymethacrylates[12,13] (for comprehensive reviews see refs 14−16). Groups have also been able to create antimicrobial nanoparticles via self-assembly mechanisms that display potent effects with minimal human cell toxicity.[5,17−21] This opens the door for the use of these agents in a new generation of biomaterials.

Generally, these synthetic polymers have been developed to suitably mimic the low molecular weight and essential amphiphilic and cationic-rich characteristics of naturally occurring AMPs. The cationic charge, stemming from the presence of high concentrations of lysine and arginine, is thought to be responsible for the initial interaction of the AMP with the negatively charged bacterial membrane, while the hydrophobic component is thought to facilitate membrane insertion and disruption.[22] To develop highly potent and selective AMP polymer mimics, one must consider and balance all aspects carefully. Much of the work to date has hence concentrated on establishing the relationships between various aspects of polymer structure and their influence on both polymer activity and toxicity. For instance, it has been shown

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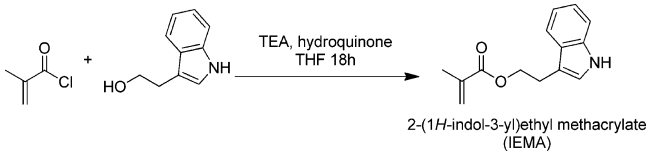
that polymer architecture,[23−25] molecular weight,[10,13,26−28] and hydrophobic−cationic balance[13,17,29−32] all appear to dramatically influence polymer activity profiles. A number of studies have also assessed the effect of substituting amine-based cationic functional groups (mimics of the amino acid lysine) with those of guanidines (mimics of the amino acid arginine).[9,13,27] There has, however, been little investigation of polymers that incorporate functional groups that mimic specific hydrophobic amino acid side chains.

AMPs have been identified that have unusually high concentrations of tryptophan (Trp) such as indolicidin, tritrpticin, and lactoferrampin.[2,33,34] A number of studies have shown Trp to have the unique ability to insert into membranes and to partition near the membrane−water interface. Moreover, its features allow it to anchor the peptide to the bilayer surface and affect lipid polymorphism.[35,36] To the best of the authors' knowledge, however, the potential benefit of the incorporation of Trp-like motifs into AMP-mimicking synthetic polymers has been left completely unexplored.

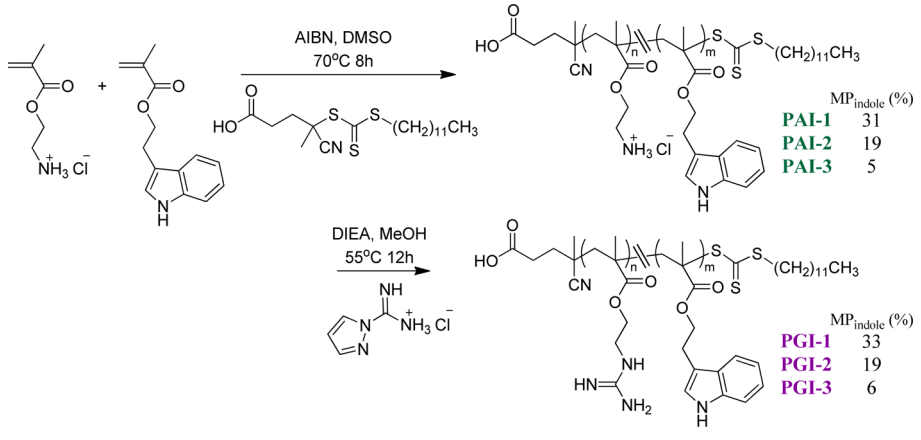
The current study details the synthesis, antibacterial, and toxicity testing of two novel series of cationic polymethacrylates which incorporate a Trp-like indole monomer, 2-(1*H*-indol-3-yl)ethyl methacrylate (IEMA, Scheme 1). Each series of amphiphilic random copolymers combines IEMA with a cationic species, either 2- aminoethylmethacrylate (AEMA) as a mimic of lysine or 2-guanidinylmethacrylate (GEMA) as a mimic of arginine. To investigate the effect of incorporation of IEMA into copolymers, the relative proportion of IEMA present was systematically varied across each series. The synthesis of IEMA (Scheme 1) was achieved in excellent yield through the esterification of methacryloyl chloride (5 equiv) with indole-3-ethanol in the presence of triethylamine base (5 equiv) and a catalytic amount of hydroquinone to prevent polymerization. IEMA was isolated using column chromatography (hexane/ethyl acetate, 4:1) before being copolymerized with commercially available AEMA using a reversible addition−fragmentation chain transfer (RAFT) process,[37,38] with the chain transfer agent (CTA) 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl] pentanoic acid and 2,2′-azobis(2-methylpropionitrile) (AIBN) as an initiator (1:5 ratio with CTA). Polymers were isolated through precipitation from methanol-ether. This afforded poly(AEMA-*co*-IEMA) 1−3 (PAI-1, PAI-2, and PAI-3) with high conversion (96−99%) and low dispersity (*Đ*) (1.14−1.17). Number average molecular weights (M_n) were calculated by ^{1}H NMR end-group analysis for both series (for details see Supporting Information and for discussion of the suitability of the method see Locock et al.[39]). *Đ* was determined by gel permeation chromatography (GPC) analysis in DMAC relative to PMMA standards. The molar percent of indole (MP_{indole} (%)) was controlled via altering the relative feed ratio of AEMA to IEMA during polymerization to give PAI-1 (MP_{indole} = 31%, M_n = 5600, *Đ* = 1.16), PAI-2 (MP_{indole} = 19%, M_n = 6300, *Đ* = 1.17), and PAI-3 (MP_{indole} = 5%, M_n = 5300, *Đ* = 1.14) (Scheme 2). The corresponding guanidine series, PGI-1, PGI-2, and PGI-3, were prepared using a postpolymerization guanylation method developed previously.[13] This allowed the retention of similar chain lengths and MP_{indole} measures for the PGI series compared with the PAI series. In short, a proportion of each of the amine PAIs was treated with 1.5 equiv of 1*H*-pyrazole-1-carboxamidine hydrochloride and 3 equiv of *N*,*N*-diisopropylethylamine relative to the number of amine units per polymer chain. The reaction was heated at 55 °C overnight under nitrogen. The resultant three poly(GEMA-*co*-IEMA) polymers (PGI-1, PGI-2, and PGI-3) were obtained via precipitation from methanol-acetone to give PGI-1 (MP_{indole} = 33%, M_n = 6000, *Đ* = 1.18), PGI-2 (MP_{indole} = 19%, M_n = 6500, *Đ* = 1.19), and PGI-3 (MP_{indole} = 6%, M_n = 5700, *Đ* = 1.15). The complete conversion from amine to guanidine pendant groups was confirmed by ^{1}H NMR analysis as previously reported.[13]

The antibacterial activity of synthesized polymers was assessed using a standard microbroth dilution assay in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines.[40] The minimum inhibitory concentration (MIC) is defined as the lowest polymer concentration to inhibit the growth of bacteria in solution and the minimum bactericidal

Scheme 1. Synthesis of the Tryptophan Mimicking Methacrylate, 2-(1*H*-Indol-3-yl)ethyl Methacrylate (IEMA)



Scheme 2. Synthesis of Random Copolymers Containing Amine-Indole Side Chains (PAI-1, PAI-2, and PAI-3 (Green)) as Lysine-Tryptophan Mimics and Guanidine-Indole Side Chains (PGI-1, PGI-2, and PGI-3 (Purple)) as Arginine-Tryptophan Mimics



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concentration (MBC) as the lowest lethal polymer concentration. Two bacterial strains were used in this study: *Staphylococcus epidermidis* (*S. epidermidis*) ATCC 35984 and methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300. The bacterial strains were selected based on their clinical relevance. *S. epidermidis* is known to rapidly form biofilms and has been identified as a major contributor to the occurrence of device-related infections (DRI).[41] The methicillin-resistant strain of *S. aureus*, with its limited treatment options, appears prevalent in nosocomial infections.[42] The toxicity of polymers was assessed utilizing human red blood cells (RBCs) using both hemolytic and hemagglutination measures. Hemolysis gives an indication of the ability of polymers to lyse RBCs and is defined as the % hemolysis observed compared to positive controls (Triton-X-100) from measures of released hemoglobin. Hemagglutination was measured through the macroscopic appearance of agglutinated cells. This agglutination behavior was rated as strong, moderate, mild, weak, or none in comparison to Concanavalin A controls.

Figures 1 and 2 show that all six polymers synthesized exhibited antibacterial activities against both *S. epidermidis* and

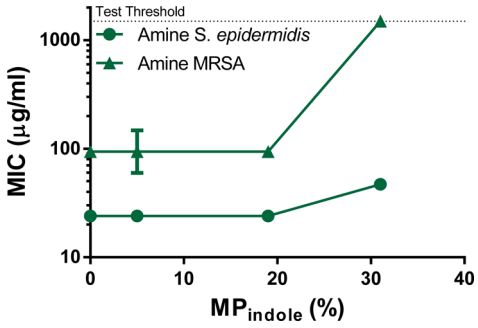

Figure 1. Antimicrobial activities (MIC) of amine polymers PAI-1, PAI-2, and PAI-3 and the cationic homopolymer p(AEMA) as a function of indole content (MP_{indole}) against *S. epidermidis* and MRSA *S. aureus*.

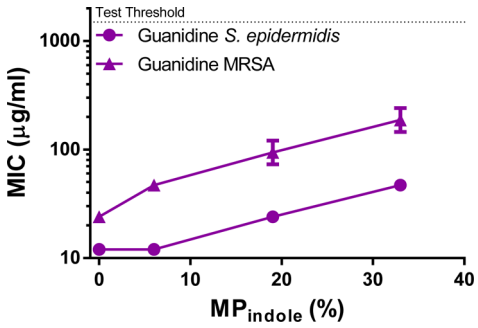

Figure 2. Antimicrobial activities (MIC) of guanidine polymers PGI-1, PGI-2, and PGI-3 and the cationic homopolymer p(GEMA) as a function of indole content (MP_{indole}) against *S. epidermidis* and MRSA *S. aureus*.

MRSA. To ascertain the effect of incorporation of indole groups, MIC and % hemolysis values obtained for two cationic homopolymers synthesized previously[13] have also been included in Figures 1 to 3 (p(AEMA) (MP_{indole} = 0%, M_n = 4000, $Đ$ = 1.15) and p(GEMA) (MP_{indole} = 0%, M_n = 3320, $Đ$ = 1.16)). MBC assays were also performed to determine if the observed MIC activity was due to a bactericidal effect of the polymers or alternatively via inhibition of bacterial growth

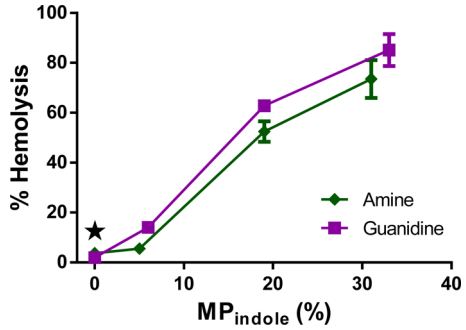

Figure 3. Hemotoxicity (% hemolysis at the equivalent *S. epidermidis* MIC) for guanidine (PAI-1, PAI-2, PAI-3, and p(AEMA)) and amine (PGI-1, PGI-2, PGI-3, and p(GEMA)) polymers as a function of indole content (MP_{indole}). The symbol '★' denotes polymers (p(AEMA) and p(GEMA)) that display moderate to strong hemagglutination.

without active cell killing. All polymers gave MBC values that were no greater than 2-fold different from their corresponding MIC values (see Supporting Information for further details). This clearly indicates the powerful bacteria-killing ability of these polymers.

Guanidine PGI-3 and the homopolymer were in particular found to be highly potent against *S. epidermidis*, both with an MIC of 12 μg/mL, and to have good potency against the methicillin-resistant strain of *S. aureus* with an MIC of 47 and 24 μg/mL, respectively. The most potent amines were PAI-3 and the homopolymer, both with MICs of 24 and 94 μg/mL against *S. epidermidis* and MRSA, respectively. The finding that guanidines appear to perform as well or better than the corresponding amine is in line with our previous work.[13] It is interesting to note that the most potent polymers identified in this study correspond to those with the lowest indole content of each series. This appears to be part of a global trend suggesting that a lower overall lipophilicity leads to increased potency. This has also been noted in our previous and ongoing work, albeit not as markedly for the guanidines.[13] This does beg the question as to whether any lipophilic component is necessary for potent and selective antimicrobial activity. While it can be seen that the cationic homopolymers are among the most active polymers, human red blood cell toxicity assays revealed them to exhibit the highest level agglutination among the group (rated as moderate to strong, for further details consult Supporting Information). Similar results have also been obtained by others.[23] Thus, it appears that some level of lipophilic character is necessary if one is to avoid such detrimental effects to human cells while maintaining adequate antimicrobial activity.

While our results suggest that some, albeit a low level, lipophilicity is favored for antimicrobial activity, there does appear to be some variance in the literature on this topic. Dogra and colleagues showed a similar trend with acrylates whereby gains in activity were observed with decreasing hydrophobicity.[43] Kuroda has shown the opposite to be the case with methacrylates,[28] as has Engler and colleagues with polycarbonates.[17] Nylon-3 copolymers[11] and polynorbornenes[10] have showed that a medium level of hydrophobicity is optimal for activity. An explanation for such observations comes to light when one considers the proposed mechanism for amphiphilic antimicrobial polymers. It is thought that cationic groups are responsible for binding to the negatively charged head groups located at the bacterial membrane.

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Following this, it is the lipophilic groups that are believed to insert into the cell membrane and bring about lysis.[30] As both aspects of polymer structure appear heavily involved in the mechanism of action, one must strike a careful balance to ensure the appropriate level of activity. It would also follow that as each polymer system denotes varying types and degrees of hydrophobic bulk or cationic character, as bestowed by each monomer, differing relationships ensue between these characteristics and observed activity.

Figure 3 depicts the observed hemolysis of the synthesized polymers versus indole content. The least hemolysis was observed with the cationic homopolymers (p(AEMA) displaying 3.7% hemolysis and p(GEMA) 1.9%) and the low-indole content copolymers, PAI-3 and PGI-3 with 5.6% and 14.0% hemolysis, respectively. This signifies that almost identical trends were observed across both series, with decreasing levels of lipophilicity giving lower levels of toxicity. This is in line with almost all studies in this area.[10,11,17,28] Taken alone, this would indicate that no lipophilic component is required for potent antimicrobial activity and low RBC toxicity. This fails, however, to consider another important aspect of RBC toxicity, that of hemagglutination. These assays showed mild to no hemagglutination across the six indole-based species, while the cationic homopolymers displayed moderate to strong agglutination behavior under the same conditions. Thus, is appears that some lipophilic component needs to be present within the polymers to mitigate agglutination-based toxicity but that this needs to be at a sufficiently low level to avoid hemolytic effects. If both aspects of hemotoxicity were not considered in this study, such trends would not have been clear.

In conclusion, we have developed facile and high yielding routes to two series of polymethacrylates inspired by the naturally occurring tryptophan-rich cationic antimicrobial polymers. Our results show that to optimize antimicrobial potency and minimize toxicity a low level of indole concentration is required for both the amine and guanidine series. Appropriate optimization of this aspect has given rise to polymers with high potency against *S. epidermidis* (PGI-3 MIC = 12 μg/mL) and the methicillin-resistant strain of *S. aureus* (PGI-3 MIC = 47 μg/mL) with minimal toxicity. Mechanistic studies will, however, be required before conclusions can be drawn around whether the indole itself plays a significant role in how these polymers interact with membranes beyond the simple donation of lipophilic bulk.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic methods, characterization, and testing methods are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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