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Inhibition of *Acinetobacter baumannii* biofilm formation on a methacrylate polymer containing a 2-aminoimidazole subunit†

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A polymeric composite containing a 2-aminoimidazole derivative was synthesized. It was found that this polymer was resistant to biofilm colonization by *Acinetobacter baumannii*, no leaching of the 2-aminoimidazole derivative was observed after 2 weeks of treatment with deionized water, and the resulting polymer was not hemolytic.

Biofilms, defined as surface attached communities of micro-organisms,[1] play a significant role driving infections of indwelling medical devices[2] (IMD). Once colonized by opportunistic bacteria, the abiotic nature of the IMD provides an ideal surface for biofilm formation. Typical treatment for IMD-related infections involve antibiotic-regimens; however, bacteria within a biofilm are approximately 1000-fold more resistant to antibiotics and are especially recalcitrant to antibiotic treatment when attached to abiotic surfaces.[3] Therefore, eradication of these infections is virtually impossible and necessitates removal of the device to stem the infection.

Given that infections of IMDs are a leading cause of hospital-acquired infections (HAIs),[4] there has been significant effort to fabricate surfaces that are resistant to biofilm formation. One of the most common practices is to impregnate the IMD with an antibiotic or biocide (such as silver);[5,6] however there are significant issues associated with efficacy lifetime, toxicity of silver to mammalian cells, and selectivity of impregnated antibiotics. These issues are further complicated by the rising occurrence of multidrug resistant bacteria in which many bacteria are resistant to antibiotics that are approved for device impregnation. Therefore, alternative methods to create materials capable of resisting biofilm formation are urgently needed.

An alternative method to impregnating devices with antibiotics/biocides is to fabricate non-leaching surfaces that resist biofilm formation through a non-microbicidal mechanism.[7,8] In this vein, we became interested in asking whether polymeric formulations that contain a 2-aminoimidazole-based antibiofilm agent would deliver a surface capable of resisting bacterial attachment. The 2-aminoimidazole (2-AI) class of anti-biofilm agents has demonstrated potent activity against both Gram-positive and Gram-negative bacteria, fungi, and mixed species biofilms.[9] Herein we report on our efforts to create methacrylate polymers that contain active 2-AI molecules and, using *Acinetobacter baumannii* as our test organism, demonstrate that these polymers effectively resist biofilm formation.

The two 2-AI molecular scaffolds that we elected to investigate for incorporation are depicted in Fig. 1. 2-Aminoimidazole/triazole (2-AIT) conjugate **1**[10] was the first compound reported to inhibit and disperse both Gram-positive and Gram-negative bacterial biofilms through non-microbicidal mechanisms while 2-AI **2**[11] has also been reported as a potent antibiofilm agent. Given that we were aiming for covalent incorporation into the methacrylate polymer, we needed to first identify methacrylate conjugates of either **1** or **2** that retained anti-biofilm activity.

We elected to first explore conjugates based upon 2-AIT **1**. This decision was based upon our previous success using the 2-AIT framework to generate a broad array of anti-biofilm compounds.[9] The two sites of methacrylate conjugation we pursued were coupling through the 2-amino position as well as through the *para* position of the β-methyl styrenyl appendage (Fig. 2). Starting with the base 2-aminoimidazole, we first acetylated the 2-amino group using acetic anhydride in trifluoroacetic acid as a solvent to determine if acyl substitutions were tolerated. Unfortunately, 2-AIT **3** only inhibited *A. baumannii* biofilm formation by $<15\%$ at 100 μM indicating that acylation abrogates anti-biofilm activity.

Next, we synthesized a 2-AIT **4**, an analogue of **1** that contained an amino methylene substituent at the *para* position. We envisioned that the primary amine could be employed as a handle for conjugation to an appropriate methacrylate derivative.[12] Unfortunately, **4** and all derivatives where the

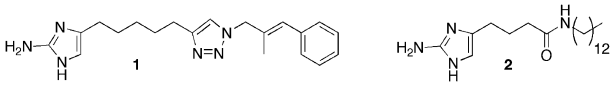

Fig. 1 2-AI-based anti-biofilm agents.

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† Electronic supplementary information (ESI) available: Synthesis and characterization data for 2-AI analogues, dose–response curves, growth curve analysis, and XPS data are provided. See DOI: 10.1039/c1cc10691k

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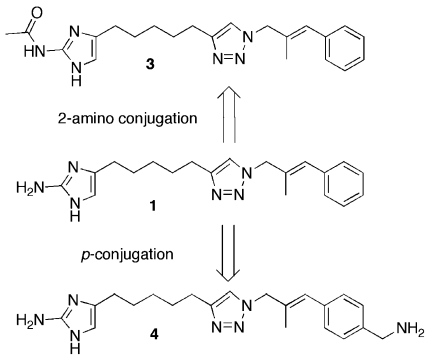


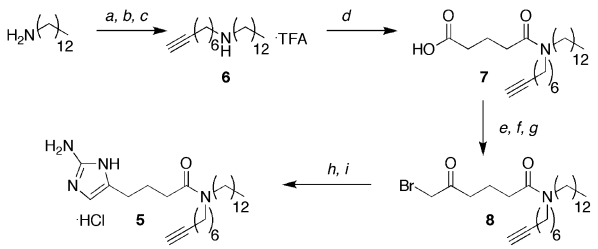
Fig. 2 Potential 2-AIT **1** analogues for methacrylate conjugation.

primary alkyl amine was functionalized also displayed poor anti-biofilm properties against *A. baumannii* (~10% at 100 μM).

Faced with the difficulties associated with obtaining appropriated functionalized derivatives of **1** that retained their anti-biofilm activity, we investigated whether derivatization of **2** would generate 2-AI analogues that retained activity. Based upon our failure with analogues of **1**, coupled with previous observations in our group that tertiary amides are tolerated within the reverse amide framework[11] and that click chemistry can rapidly functionalize 2-AI derivatives,[9] we focused on generating tertiary amide analogues of **2** that could rapidly be appended to methacrylate *via* click chemistry. With this goal, we identified reverse amide **5** as a potential conjugate for evaluation.

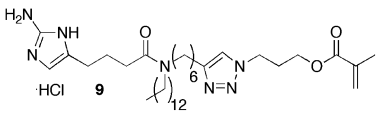
Compound **5** was synthesized as outlined in Scheme 1. Tridecylamine was Boc-protected (Boc_2O/CH_2Cl_2), *N*-alkylated with 8-iodo-1-octyne that, after removal of the Boc group (TFA/CH_2Cl_2), delivered secondary amine **6**. Amine **6** was then employed in an aminolysis reaction with glutaric anhydride to generate carboxylic acid **7** that was then converted to α-bromo ketone **8** by conversion to the corresponding acid chloride (oxalyl chloride/cat. DMF/CH_2Cl_2) followed by reaction with diazomethane and quenching with HBr. Finally, the 2-aminoimidazole was installed by condensation of **8** with Boc-guanidine[13] that, following Boc-deprotection and counterion exchange, delivered **5**.

Compound **5** was initially screened, in solution, for the ability to inhibit *A. baumannii* biofilm formation using the crystal violet reporter assay.[14] Initial inhibition studies indicated that **5** inhibited biofilm formation by >95% at 100 μM, while



Scheme 1 Synthesis of **5**. (a) Boc_2O, DCM, 0 °C to rt, 1 day, 98%; (b) NaH, 8-iodooct-1-yne, DMF : PhMe (2 : 1), rt, overnight, 94%; (c) DCM : TFA (2 : 1), 0–rt, 3 h, quant.; (d) glutaric anhydride, DMAP, Et_3N, DCM, rt, 2 days, 96%; (e) $(COCl)_2$, DMF(cat.), DCM, −20 °C–rt, 2 h; (f) CH_2N_2, Et_2O, DCM, 0 °C, 1 h; (g) HBr, 0 °C, 0.5 h, 46% over 3 steps; (h) Boc-guanidine, DMF, rt, 3.5 days, 62%; (i) DCM : TFA (2 : 1), rt, 2 h, then MeOH, HCl (conc.), quant.

dose response studies revealed that **5** inhibited *A. baumannii* formation with an IC_{50} of 13 μM. Follow up analysis of *A. baumannii* grown in the presence of 13 μM **5** indicated no defect in the growth curve in comparison to bacteria grown in the absence of **5**, indicating that the 2-aminoimidazole is modulating biofilm formation through a non-microbicidal mechanism. We subsequently synthesized the 2-AI/methacrylate conjugate **9** *via* click chemistry (ESI†) and assayed for its ability to inhibit biofilm formation. Paralleling the results with **5**, conjugate **9** inhibited biofilm formation by >95% at 100 μM. Follow up dose response studies and growth curve analysis revealed that **9** inhibited *A. baumannii* formation with an IC_{50} of 17 μM and was nontoxic to the bacteria.



With an active methacrylate conjugate in hand, we then turned to fabricating methacrylate co-polymers that contained **9**. The methacrylate co-polymer film was synthesized by the photo-polymerization of a formulation containing 4 wt.% **9**, 35% isobornyl methacrylate co-monomer, and 59% polyurethane methacrylate (PUMA) crosslinker using 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone at a concentration of 2.0 wt.% as the photoinitiator.[15] The formulation was exposed to a 450 W broadband UV lamp for six minutes between two planes of glass. Energy curable PUMAs are common in the construction of IMDs[16] and provide these desired properties, therefore this was an appropriate model to study the efficacy of **9** grafted to a polymer surface.

The synthesis and characterization of the PUMA is outlined in the ESI.† To validate the presence of **9** in the polymer, matrix infrared spectroscopy and ^{13}C solid state NMR were both conducted; however the results were inconclusive owing to the difficulty in distinguishing between the blank and the polymer with 4% **9** (designated **P9**). We then turned to X-ray photoelectron spectrometry (XPS), which proved to be more fruitful. Samples were soaked in DI water for 24 hours to remove any non-covalently bonded material, rinsed, and then dried under reduced pressure. We examined the samples for changes in the carbon (1s), nitrogen (1s), and oxygen (1s) content and type between the blank and **P9**. We found a marked increase in the nitrogen content (1.7% to 7.5%) along with the emergence of 0.7% chloride (2p) not found in the blank, both attributable only to the presence of the 2-amino-imidazole HCl salt within the polymeric matrix.

Once fabricated, polymeric strips (both blank strips and **P9**) were challenged with either media only or media containing *A. baumannii* for 24 hours. After 24 hours, media containing bacteria had reached the stationary phase (Fig. 3A) and each polymeric strip was removed and washed thoroughly with water to remove any loosely adherent bacteria. Each strip was then stained with crystal violet to visualize the remaining bacterial mass that was attached (Fig. 3B). The resulting crystal violet was then solubilized with ethanol and quantified spectrophotometrically (A_{540}). In comparison to the blank strips, **P9** showed *ca.* 85% reduction in attached bacteria. We then monitored bacterial growth in the absence or

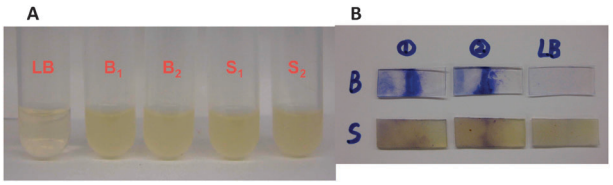


Fig. 3 B = control polymer, S = **P9**. Subscripts refer to different samples. (A) Visualization of bacterial growth after 24 hours when challenged with polymeric composites. LB = media only. OD_{600} of B_1/B_2 = 3.07, S_1/S_2 = 3.06. (B) Results of crystal violet staining after polymers were exposed to bacteria or media for 24 hours.

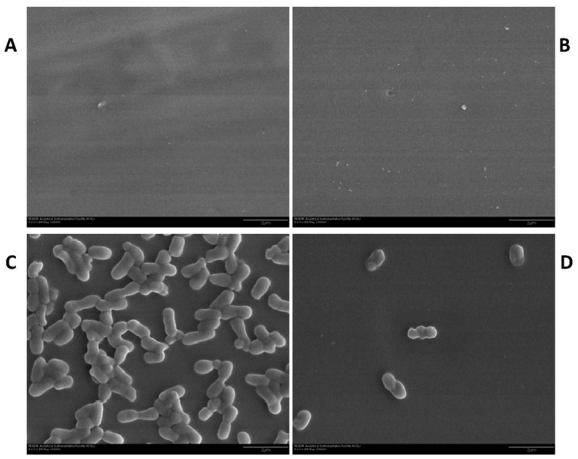


Fig. 4 SEM images. (A) Blank polymer. (B) **P9**. (C) Blank polymer exposed to bacteria for 24 hours. (D) **P9** exposed to bacteria for 24 hours. Images are 10 000× magnification.

presence of polymeric strips for 24 hours (both blank strips and **P9**) and observed no difference in bacterial growth, confirming that the reduction in bacterial attachment for **P9** was due to a non-microbicidal mechanism.

Given that the crystal violet assay: (1) only reports on the amount of biomass attached to the surface and (2) may result in non-specific staining, we wanted to further analyze the polymer surfaces for biofilm inhibition. To achieve this, blank polymers and **P9** were again exposed to *A. baumannii* for 24 hours. The resulting strips were then washed and imaged using scanning electron microscopy (Fig. 4). Blank polymers allowed biofilm development, while we only observed a few individually attached bacteria lacking any community structure on **P9**, clearly demonstrating that **P9** efficiently inhibited biofilm development on its surface.

After establishing that **P9** retained the antibiofilm activity of its co-monomer **9**, we ran a number of experiments to determine how robust the polymer was to various conditions that would promote leaching of any non-covalently bound **9** and erode activity. **P9** continually washed with distilled water for up to 14 days (longest time tested) and then exposed to *A. baumannii* for 24 hours demonstrated minor reduction in antibiofilm activity (*ca.* 75% reduction assessed by the crystal violet assay). We then performed leaching experiments with either methanol or hexanes for 24 hours. After solvent treatment, **P9** still inhibited biofilm development by *ca.* 75%. We then analyzed both the methanol extract and hexane extract by LCMS and were only able to detect the initiator from the polymerization reaction.

We also probed the importance of co-fabricating the polymer with a 2-AI/methacrylate conjugate by substituting **5** for **9** in the polymerization reaction. The resulting polymeric composite inhibited biofilm formation on its surface by *ca.* 85%. However, leaching the polymer in methanol for 24 hours abrogated all anti-biofilm activity.

Finally, we tested the hemolytic potential of **P9**. Control methacrylate polymer or **P9** was incubated in the presence of red blood cells. Both polymers showed no detectable hemolysis, equivalent to cells treated with phosphate buffered saline.

In conclusion, we have demonstrated that a 2-AI/methracrylate conjugate retains its anti-biofilm activity when incorporated into a methacrylate co-polymer. These polymers show only minor reduction in activity under conditions designed to remove non-covalently bound monomers. Current efforts in our laboratory now focus on optimizing these polymeric materials to achieve greater biofilm inhibition capabilities and evaluating their efficacy *in vivo*.

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