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Guanidinium-Functionalized Photodynamic Antibacterial Oligo(Thiophene)s

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ABSTRACT

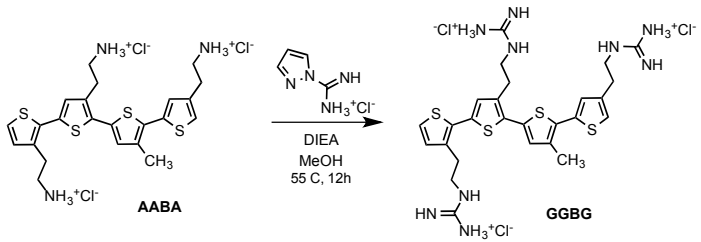
We synthesized precision oligomers of thiophene with cationic and hydrophobic side chains to mimic the charge, hydrophobicity, and molecular size of antibacterial host defense peptides (HDPs). In this study, the source of cationic charge was a guanidinium salt moiety intended to reflect the structure of arginine-rich HDPs. Due to the pi-conjugated oligo(thiophene) backbone structure, these compounds absorb visible light in aqueous solution and react with dissolved oxygen to produce highly biocidal reactive oxygen species (ROS). Thus, the compounds exert bactericidal activity in the dark with dramatically enhanced potency upon visible light illumination. We find that guanylation of primary amine groups enhanced the activity of the oligomers in the dark but also mitigated their light-induced activity enhancement. In addition, we also quantified their toxicity to mammalian cell membranes using a hemolysis assay with red blood cells, in the light and dark conditions.

INTRODUCTION

Antibacterial agents are increasingly in high demand due to the rise of antibiotic resistance in pathogenic bacterial infections, combined with the decreasing number of new antibiotic drug approvals.[1] Host defense peptides are one alterative approach to combat infectious disease.[2] These peptides are components of the innate immune system in all multicellular organisms and they putatively kill bacteria by either by a direct mechanism that involves physical disruption of the bacterial cell membrane or by indirect immune-modulatory effects.[3-5] The membrane disruption ability is due to their cationic and amphiphilic structures, which facilitate binding to anionic components of the bacterial cell envelope as well as insertion into the hydrophobic membrane core.[6]
Synthetic oligomers and polymers, primarily composed of cationic and hydrophobic groups, have been widely studied as mimics of HDP structure and function.[7] The chemical structure of cationic groups in these polymers/oligomers plays a key role as a determinant of biological activity.[8-9] Whereas the majority of HDP-mimicry has focused on polymers with primary amines or quaternary ammonium salt (QAS) groups as the

source of cationic charge,[10] many HDPs are rich in cationic arginine residues, which display pendant guanidine groups. As such, several groups have investigated guanidinium-containing synthetic polymers as a strategy to enhance the binding to anionic membranes, for applications as antibacterials and as delivery vehicles.[11-14] Indeed, the guanidine group is known to complex strongly with anionic phospholipids due to a combination of Columbic attraction and bidentate hydrogen bonding interactions.[15-16]

Photodynamic antibacterial therapy is becoming an increasingly examined area of research.[17] In short, cationic π-conjugated compounds bind to bacterial cells and then produce reactive oxygen species (ROS) upon excitation with light, thereby leading to cell death by oxidative stress pathways.[18] Whitten and Shanze,[19-25] as well as other groups,[26-28] have widely reported on π-conjugated oligomers and polymers, typically bearing quaternary ammonium salt (QAS) and imidazolium groups as the course of cationic charge. There remains a relative paucity of studies on π-conjugated oligomers and polymers with HDP-mimetic structures, in terms of the source of cationic charge (amines in Lysine and guanidines in Arginine residues) as well structural elements like de novo design of facially amphiphilic conformations. The combination of HDP-mimetic molecular design with the photodynamic therapy approach is therefore of current interest in our group. We recently developed cationic and amphiphilic pi-conjugated oligomers are attractive that kill bacteria locally upon visible light irradiation.[29] These technologies may lead to rapid topical therapies to cure bacterial infections that can be accessed by a light source, which circumvents concerns about toxicity to surrounding tissues that are not irradiated. Whereas our first generation library focused on primary amines as the source of cationic charge, here we focus on the transformation of the amines into guanidines and the resulting impact on the structure-activity relationships.



Scheme 1. Synthetic route used to convert the primary amine groups in compound AABA to guanidinium groups in GGBG, an olgio(thiophene) bearing a +3 cationic charge at neutral pH.

EXPERIMENTAL

Synthesis.

The synthesis of the precursor oligo(thiophene), and their functional derivatives bearing primary amine groups, were previously reported by our group.[29-30] In this work, we further modified this molecule to convert the amine groups to guanidine groups, which yielded the new compound GGBG (Scheme 1). For this work, the precursor compound bearing primary amine side chains, AABA (8 mg, 0.018mmol), N,N-diisopropylethylamine (DIEA, 16.5mg, 0.16mmol) and 1H-Pyrazole-1-carboximidamide hydrochloride (12 mg, 0.08 mmol) were dissolved in methanol (1 mL) in a round-bottom glass flask (5 mL). The reaction mixture was stirred at 55 °C

overnight. After stirring overnight, the crude product mixture was then purified by passing through a column loaded with LH-20 size-exclusion gel (eluent: 100% MeOH) to obtain the pure product as an orange powder (4.2 mg, 41% yield). ^{1}H NMR (500 MHz, MeOH-d_4): δ = 7.46-7.10 (m, 6H), 3.58-3.55 (m, 6H), 3.17-3.14 (m, 4H), 2.99-2.96 (t, 2H), 2.44 (s, 3H). HRMS (ESI, m/z): [M−2H$^+$ = $C_{26}H_{34}N_9S_4^+$] Calc. 600.1815, Found. 600.1803.

Antibacterial Activity

The minimum inhibition concentration (MIC) of the compounds in this work were defined as the lowest concentration required to completely inhibit the growth of E. coli in nutrient-rich Muller-Hinton broth (5×10^6 CFU/mL, OD_{600} = 0.001). Oligo(thiophene) solutions were prepared as 2-fold serial dilutions from a DMSO stock (5 mg/mL). A single colony of E. coli ATCC 25922 was inoculated in Muller-Hinton (MH) broth at 37 °C in a shaking incubator overnight. The turbid suspension was diluted to OD_{600} = 0.1, regrown for 90 min (OD_{600} = 0.5 – 0.6), and then diluted to OD_{600} = 0.001 in MH broth. The bacterial suspension (90 μL) was mixed with each polymer concentration (10 μl) in a sterile 96-well round-bottom polypropylene microplate. The microplate was incubated for 16 hours at 37 °C either in dark or light. Incubation under visible white light illumination was performed using a common desktop reading lamp (3.55 W LED bulb, distance 5 cm, ~320 lumens). All set of polymers were tested twice, each in duplicate, on different days. As a positive growth control, dilutions of DMSO with no compound were prepared and tested in the same manner with E. coli. As a negative growth control (for sterility), MH broth alone with no bacteria was also tested in triplicate.

Hemolytic Activity

Hemolytic activity of the oligo(thiophene) was determined by a standard hemoglobin release assay, using the same serial-dilutions of compound that were used for the MIC tests. First, 1 mL of 10% (v/v) sheep red blood cells was centrifuged at 1000 rpm for 5 min and washed with PBS, pH 7.4, and this procedure was repeated twice. The resulting stock was diluted 10-fold in PBS to provide 1% (v/v) RBC assay stock. In a sterile 96-well round-bottom polypropylene microplate, 90 μl of 1% (v/v) RBC assay stock was mixed with 10 μl of each of the oligothiophene serial dilution. PBS was the negative control and 0.1% Triton X-100 (v/v) was the positive lysis control. The microplate was secured in orbital shaker at 37 °C and incubated at 180 rpm for 60 min. The microplate was then centrifuged at 1000 rpm for 10 min. In another sterile microplate, 10 μl of supernatant was diluted in 90 μl PBS. The absorbance at 415 nm was recorded using a microplate reader. Hemolysis was plotted as a function of polymer concentration and the HC_{50} that is described as the polymer concentration causing 50% hemolysis relative to the positive control. This value was estimated by the fitting the experimental data to the Hill equation, $H = 1/(1+(HC_{50}/[c])^n)$, where H is the hemolysis fraction ($H = [OD_{415}(\text{polymer})-OD_{415}(\text{buffer})]/[OD_{415}(\text{TritonX})-OD_{415}(\text{buffer})]$), [c] is the concentration of compound, n and HC_{50} are the curve fitting parameters. All experiments were repeated twice, each in duplicate, on different days. The absorbance values from each trial were averaged and then the HC_{50} was calculated.

RESULTS AND DISCUSSION

Synthesis and Characterization

We successfully converted all primary amine groups of AABA into guanidium salts to yield the product GGBG in fair yield (41%). Most of the mass lost is due to the purification by preparative size-exclusion chromatography on LH-20 gel, which was required to completely remove the large excess of the carboximidamide guanylation reagent. This procedure may be optimized in the future with a better resolution gel, or perhaps by dialysis, to improve purification efficiency and likely approach quantitative yield. The resulting compound is readily soluble in polar organic solvents (e.g. DMF, DMSO) and aqueous media up to at least 10 and 1 mg/mL, respectively, which were the highest concentrations we tested. The solubility data are fully amenable to the biological assays described below, since the typical concentration range for testing MIC and HC50 are in the μg/mL range. The ^{1}H NMR spectrum of GGBG (Figure 1) is in excellent agreement with the proposed chemical structure. Aromatic protons attributable to the thiophene backbone appear in the 7.0-7.5 ppm region, as expected. The methylene groups adjacent to the thiophene units appear as resonances at 3.2 ppm for the α and β units (labeled x in Figure 1) whereas the corresponding methylene on the terminal δ unit appears at 2.9 ppm (labeled x* in Figure 1). The lone methyl side chain on the γ thiophene unit appears at 2.4 ppm, as expected.

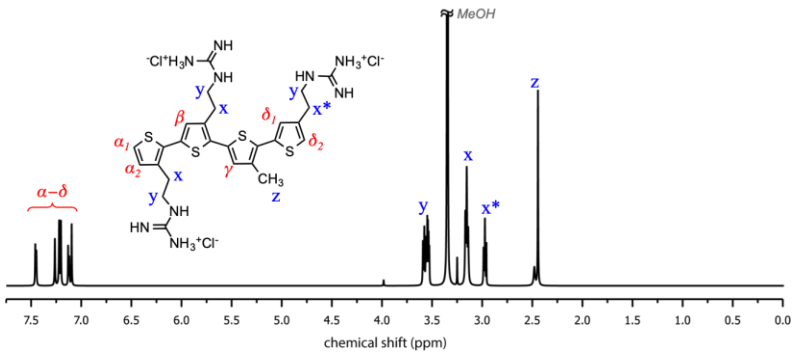


Figure 1. ^{1}H NMR spectra in deuterated MeOH for compound GGBG with peak assignments given.

We proceeded to further confirm the chemical identity of GGBG using electrospray ionization (ESI) high-resolution mass spectroscopy (HRMS) in positive ion mode (Figure 2). The compound was observed in the mass spectra as the molecular ion minus two protons [$M^{3+}-2H^+$], which has a net charge of z = +1. Thus, the theoretical exact molar mass is m/z = 600.1815. The major observed peak observed experimentally in the ESI HRMS appears prominently at m/z 600.1803, an excellent match.

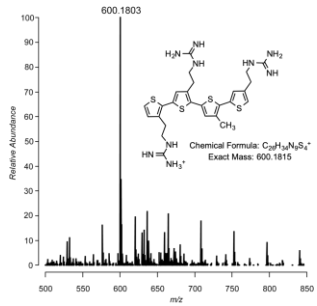


Figure 2. Electrospray ionization (ESI) high-resolution mass spectroscopy (HRMS), positive ion mode.

Absorbance and Fluorescence.

Next, we examined the absorbance and emission spectra for AABA and GGBG in dilute MeOH solution. These compounds both exhibit a broad, featureless absorbance peak centered at 360 nm, which extends only weakly into the visible range up to about 500 nm. The emission spectra for both compounds centered around 480 nm, with two maxima near 460 and 490 nm. In both absorbance and emission, it seems that the peaks for GGBG are slightly broader than for AABA, which is perhaps related to subtle differences in conformational flexibility arising from the extent of charge-charge repulsion between the pendant cationic side chains. Overall, the optical properties of these two compounds appear to be very similar, which implies that the backbone oligo(thiophene) structure dominates and that the side chain chemistry does not significantly impact the photophysical properties of these molecules in dilute MeOH solution. Subtle differences in optical properties may influence the photodynamic biological activity. Ongoing work will include measurements of absorbance and emission when these molecules are bound to the interface between the aqueous phase and a model lipid bilayer.

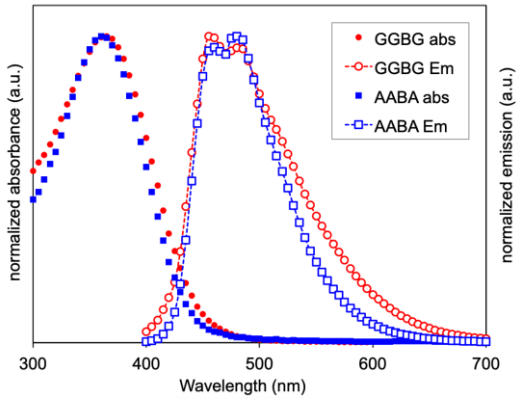


Figure 3. Absorbance and emission spectra for AABA and GGBG.

Biological Activity

The antibacterial activity is defined here as the minimum inhibitory concentration (MIC), which is the lowest concentration of compound that completely inhibits the growth of *E. coli* cells (10^6 CFU/mL) upon incubation in nutrient-rich MH growth media for 18 hours at 37 °C, either in the dark or under visible light illumination with a 3.55 W LED bulb at a distance of 5 cm (320 lumens).

In the dark, the guanylated compound GGBG exhibited inhibitory activity that is evocative of a highly potent antibacterial agent (MIC_{dark} = 1.6 μg/mL), with activity that surpassed the precursor amine-containing compound AABA (MIC_{dark} = 4 μg/mL) For comparison, the bee venom toxin peptide melittin, which is widely considered a highly potent membrane-disrupting biocidal agent,[31] gave an MIC value of 4 μg/mL in the same assay conditions.

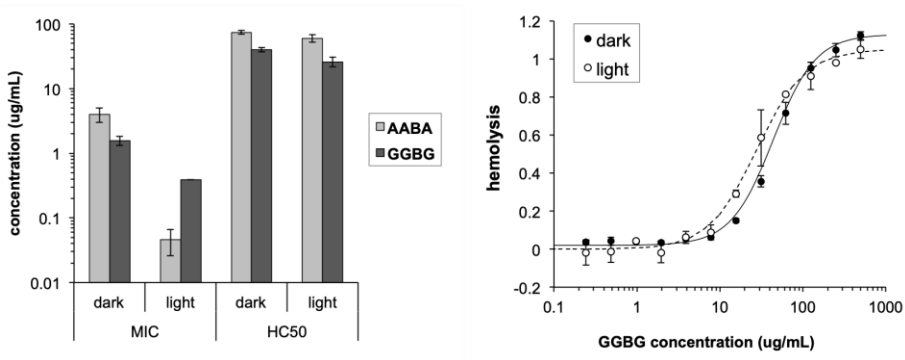


Figure 4. Antibacterial and hemolytic activity, in both dark and light incubation conditions, for GGBG and AABA. Hemolysis dose-response curves (right panel), in the light and dark, as a function of GGBG concentration. Curve fits were performed using the modified Hill equation.

Under visible light illumination, the MIC values for both AABA and GGBG are enhanced, but to differing extents. As reported previously,[29] the antibacterial activity of AABA is markedly improved from MIC_{dark} = 4 μg/mL to MIC_{light} = 0.046 μg/mL, a nearly ten-fold enhancement due to the photodynamic effect. On the other hand, the photodynamic enhancement for GGBG was more modest: a relatively modest improvement in antibacterial activity from MIC_{dark} = 1.6 μg/mL to MIC_{light} = 0.39 μg/mL was observed. Thus, guanylation of amine groups in these cationic, amphiphilic oligo(thiophene)s appears to improve activity in the dark, but apparently mitigates the photodyanmic enhancement of activity under visible light illuination. The purpose of guanylation of the side chains was to enhance the membrane-targeting activity of these oligomers, which has been confirmed previously in other non-photoactive systems but has never been investigated in the context of photodynamic activity. Unfortunately, it would seem that the benefit of guanylayion (improved activity in the dark) is outweighed in this case by the much less pronounced photodynamic enhancement of activity exerted by GGBG. Thus, we find that guanylation may not be an effective molecualr design principle for photodyanmic oligomers, in contrast to previously studied membrane-disrupting antibacterial polymers/oligomers.

Both AABA and GGBG are similarly hemolytic, both in the light and dark conditions. The $HC_{50,dark}$ values are 74 and 40 μg/mL, respectively, which implies that both are somewhat hemolytic but at much higher effective concentrations than their antibacterial activity. The ratio of HC_{50}/MIC is a commonly used metric to assess cell-type selectivity in this field. In the dark, AABA and GGBG exhbitied HC_{50}/MIC ratios of 19 and 25×, respectively. For comparison, the toxic peptide melittin has an HC50 value of 6 μg/mL, which gives a selectivity ratio of just 1.5×, corresponding to essential indicriminate biocidal behavior. Under visible light, the HC_{50} values of AABA and GGBG both decreased to a minor extent (60 and 26 μg/mL, respectively). Owning to the extremely potent photodyanmic enhancement in the case of AABA, this compound has a HC_{50}/MIC ratio of 1304× under visible light. On the other hand, GGBG shows a relatively modest improvement in cell-type selectivity upon visible light illumination, giving a HC_{50}/MIC ratio of 66×. The hemoglobin release dose-response curves for GGBG are also given in Figure 4, right panel. The Hill curve-fitting exponents are $n = 1.7$ and 1.5 in the dark and light, respectively. For both cases, exponents near unity imply that these compounds do not likely exert their lytic mechanism in a cooperative manner.

CONCLUSIONS

We report the successful functional group conversion of primary amines to guanidine groups in the side chains of oligomeric tetrathiophene compounds. These compounds are water soluble, cationic/amphiphilic, and photodyanmic in nature. Both the amine- and guanidine containing oligo(thiophene)s are highly potent antibacterial agents in the dark and show enhanced antibacterial activity under visible light illumination with a weak white source (a simple desktop reading lamp). By comparison, the primary amine containing compound showed much stronger photodynamic enhancement relative to the guanidine-functionalized counterpart. The hemolytic toxicity of these compounds is moderate, with good cell type selectivity in the dark. In the light, hemolytic toxicity is only slightly aggravated, which leads to improved cell-type selectivity in both cases. Studies on the mechanism of the antibacterial activity and the toxicity to human cells, both in the light and dark conditions, are fertile areas for future research in this field.

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