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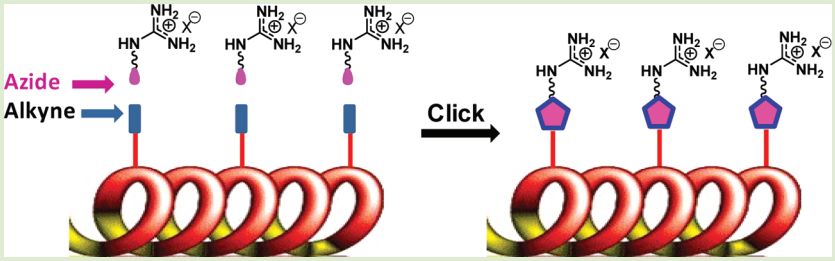
Synthesis of Guanidinium Functionalized Polycarbodiimides and Their Antibacterial Activities

Januka Budhathoki-Uprety,[†] LingLing Peng,[†] Christian Melander,[†] and Bruce M. Novak*[,‡]

[†]Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States

[‡]Department of Chemistry and the Alan G MacDiarmid NanoTech Institute, University of Texas at Dallas, Richardson, Texas 75080-3021, United States

Supporting Information



ABSTRACT: A family of guanidinium-side-chain functionalized polycarbodiimides has been synthesized by allowing an azido guanidinium salt to react with alkyne polycarbodiimides via the copper catalyzed [3 + 2] cycloaddition (Click) reaction. **Poly-2(a–d)** are cationic/amphiphilic polymers in which the global hydrophilic/hydrophobic balance has been tailored by local alteration of the length of alkyl side chain in the repeat unit of polymers prior to polymerization. The shorter alkyl chains yield water-soluble polymers, **Poly-2c**, **-2d**, and **-2e**. Antibacterial activities of these cationic polycarbodiimides have been investigated for Gram-positive and Gram-negative bacteria that include *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, and *Acinetobacter baumannii*. It was observed that the influence of hydrophobic–hydrophilic balance per repeat unit of these polymers have profound effects for both antimicrobial and hemolytic activities. In addition, these polycarbodiimide-guanidinium-triazole conjugates offered moderate to significant antibacterial activity and rapid interaction with red blood cells causing blood precipitation without significant hemolysis in case of **Poly-2(b–e)**. This latter property has the potential to be exploited in the polymer coatings or wound protection.

Natural antimicrobial peptides are known to kill broad spectrum of bacteria without significant bacterial resistance.[1] Antimicrobial activity in natural host defense peptides is believed to originate from their cationic and hydrophobic residues that help fold peptides into amphiphilic secondary structure upon binding to biomembranes.[2] Difficulties associated with the sequence controlled synthesis of such antimicrobial peptides in large quantities limits their accessibility and use for therapeutic applications. Alternatively, a number of synthetic mimics of antimicrobial peptides using conventional polymers systems such as polystyrenes,[3] polyvinylpyridines,[4] polyacrylates, oligoarylamides,[5] and polynorbornenes[6] have been prepared and their potential as antimicrobial agents have been evaluated. In these synthetic mimics, the amphiphilic nature, overall hydrophobic–hydrophilic balance, and charge density have been found crucial in developing better polymeric antimicrobial compounds.[6,2,7] Natural antimicrobial peptides contain basic side chains (e.g., primary amine in lysine and guanidine in arginine) that are protonated at physiological pH generating net positive charges. Inspired from nature, cationic amphiphilic macromolecules, in particular, dendrimers, oligomers, and polymers with guanidine moieties either in the backbone[8] or in the side chains,[9,10] have been synthesized and reported as therapeutic agents such as polycationic gene delivery agents, antimicrobial compounds, stimuli responsive anion transporters,[11] molecular transporters,[12] and so on. For antimicrobial polymers, influence of hydrophobic/hydrophilic balance, molecular weight, the positive charge density, its distribution along the polymeric chain, pK_a of ionizable groups, and the three-dimensional structure and flexibility of polymer backbone all have been studied and found to play key roles in governing biological activities.[2,6,13] As such, a synthetic polymer with a stable polymer backbone and tunable pendant groups facilitates the design of such new polymeric therapeutic materials.

Polycarbodiimides are helical polymers synthesized from carbodiimide monomers using transition metal catalysts in a living fashion.[14–16] Their nitrogen-rich polymer backbone is resistant toward enzymatic degradation and the two tunable side chains per repeat unit may bring immense potential as scaffolds for the design of polymeric therapeutic agents. We previously reported a family of alkyne polycarbodiimides and

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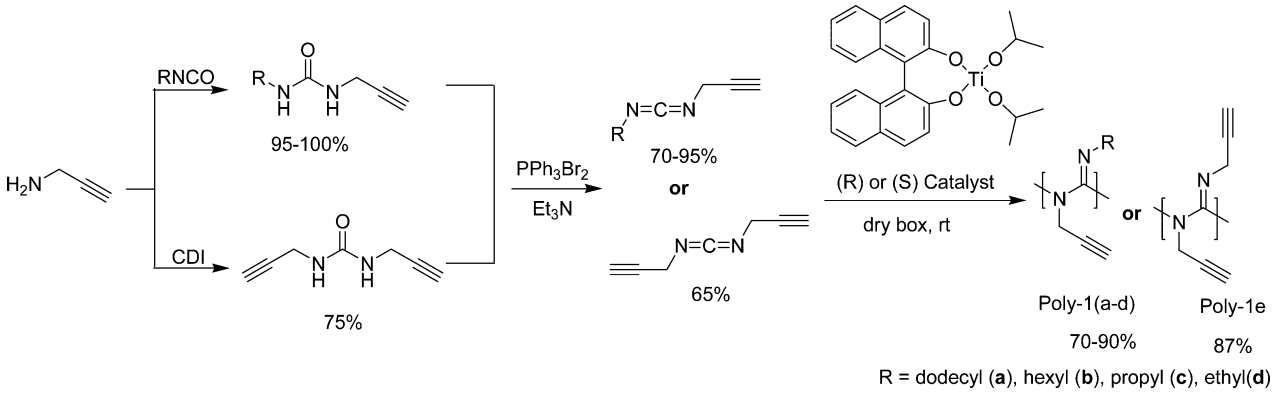
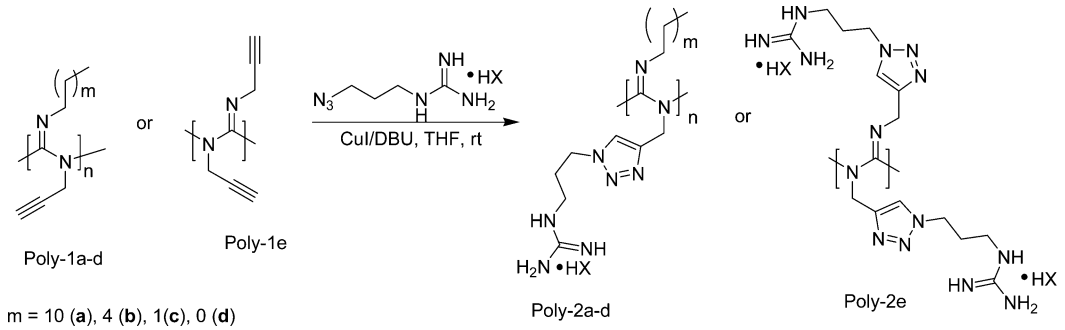
Scheme 1. Synthesis of Alkyne-Functionalized Polycarbodiimides: Precursor Polymers



Scheme 2. Modification of the Polycarbodiimide Side Chains via Click to Incorporate Guanidinium Side Chains



their post modification by copper-catalyzed alkyne−azide cycloaddition.[17] In this research work, we utilize alkyne ligation handles of the alkyne-substituted polycarbodiimides to incorporate guanidine functionality into the polymer system. Successful introduction of a biologically relevant pendant group, guanidine, which, in some cases, act superior than quaternary ammonium groups in synthetic antibacterials,[6] inspired us to study antibacterial activities and hemoreactivity in these new polymers. To the best of our knowledge, this is the first report on bioactivity study in such polycarbodiimides. Here, we focus on Gram-negative *E. coli* and *A. baumannii* and Gram-positive *S. aureus* and methicillin-resistant *S. aureus* (MRSA), mainly due to their predominant roles in hospital-acquired infections.[18,19] Gram-positive bacteria are the commonest cause of nasocomial infections[18] with *S. aureus*, in particular, MRSA, being reported as a leading cause of hospital-acquired and community-acquired infections.[20,21]

Alkyne-functionalized precursor polymers, **Poly-1(a−e)** were synthesized in three steps from the commercial amine and isocyanates (Scheme 1). The carbodiimide monomers were obtained in excellent yield from the corresponding urea derivatives.

The coordination-insertion polymerization of the carbodiimide monomer using titanium(IV) catalyst performed at room temperature can easily be monitored by disappearance of a very strong IR band at around 2130 cm^{-1}, corresponding to N═C═N. A new IR band at around 1640 cm^{-1} corresponding to imine in the polymer backbone emerges as polymerization proceeds. Once the polymerization was complete, the resulting orange yellow solid was dissolved in chloroform (or THF) and precipitated in methanol, filtered and dried to afford pale yellow solid. The resulting polymers were characterized by ^{1}H NMR, ^{13}C NMR, and FTIR. Size exclusion chromatography (SEC) performed on these polymers using polystyrene standards to determine the relative molecular weights showed **Poly-1a** (M_n = 24000 Da, PDI = 3.20), **Poly-1b** (M_n = 14000 Da, PDI = 2.19), and **Poly-1c** (M_n = 1400 Da, PDI = 2.60). SEC was not performed on **Poly-1d** and **Poly-1e** due to their limited solubility in eluent (THF/diethanolamine). Due to strong affinity and adhesion between polycarbodiimides and SEC column matrix materials[15−17,22] and rigid rod structure of polycarbodiimides, molecular weight, and particularly the PDI determinations of polycarbodiimides using SEC are relatively less reliable when comparisons are made with random coil polystyrene standards.

The hydrophobicity of the polymers was tailored in **Poly-1(a−d)** by altering the length of alkyl group in the corresponding monomers prior to polymerization. In **Poly-1(a−d)**, repeat unit provides one terminal alkyne pendant and long or short aliphatic hydrophobic tail, whereas **Poly-1e** offers two terminal alkyne pendants per repeat unit which doubles potential sites for post modification. Along the stiff, helical polycarbodiimide backbone the terminal alkyne pendants are exposed and are accessible for postpolymerization modification.

Poly-1(a−e) were then decorated with guanidinium pendants via a covalent triazole linker resulted from alkyne−azide click reaction (Scheme 2) converting the repeats into cationic amphiphilic moieties.

Spectroscopically, the incorporation of guanidinium side chains into the polymer system has been confirmed by ^{1}H NMR signals appearing at 6.8−8.2 ppm for NHs from guanidinium unit (Figure S1 in the SI) and in ^{13}C NMR a resonance at 158 ppm corresponding to the guanidinium carbon. Also appearing are additional broad IR signals around 3300−3400 cm^{-1} corresponding to the presence of N−H stretches; a strong band around 1658 cm^{-1} corresponding to

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the guanidine imine. Consistent with our previous publication[17] the degree of functionalization by click chemistry is again quantitative as reveled by IR spectroscopy (spectra shown in SI). SEC was not performed after click reaction on these polymers due to insolubility of resulting polymers in eluent (THF/diethanolamine). However, the polymer samples were dialyzed in a dialysis membrane with molecular weight cut off 35K prior to activity tests to ensure sufficiently high molecular weight.

With guanidinium-functionalized polymers, **Poly-2(a–e)**, on hand, we then tested for antibacterial activity against *E. coli*, *A. baumannii*, *S. aureus*, and MRSA using the broth microdilution technique. Each test was performed in duplicate and repeated at least twice. The length of the alkyl chain in the repeat unit showed a great influence on antibacterial activity of these polymers (Figure 1). The more hydrophobic polymer, **Poly-2a** with long dodecyl side chain in the repeat unit has little or no effect on all tested bacteria up to concentrations of 1024 μg/mL. Decreasing the alkyl chain length in the repeat unit to increase the overall hydrophilicity and cationic charge density in the polymers resulted into increased antibacterial activities. The polymers in this study, **Poly-2(b–e)** demonstrated better antibacterial activities against Gram-positive bacteria *S. aureus* and MRSA than Gram-negative bacteria *E. coli* and *A. baumannii*. Minimum inhibitory concentration (MIC, i.e., the concentration of the sample to show no visible bacterial growth in the test solution) of **Poly-2b** against *S. aureus* is 64 μg/mL, whereas that for **Poly-2(c–e)** is 128 μg/mL. For MRSA, **Poly-2b** and **-2c** each show MIC 128 μg/mL, whereas **Poly-2d** has MIC 128 μg/mL (slight fluctuation noticed in different runs) and **Poly-2e** was found to be the most effective one, displaying a MIC 64 μg/mL. The MIC for *E. coli* is 64 μg/mL for **Poly-2d**, 256 μg/mL for **Poly-2b**, and 512 μg/mL for **Poly-2e**. **Poly-2c** showed MIC 128 μg/mL with slight fluctuations in different runs. *A. baumannii* is found to be more resistant toward these polymers. **Poly-2a**, **-2b**, and **-2e** did not kill the bacteria (*A. baumannii*) under the maximum tested concentration, 1024 μg/mL, **Poly-2d** showed MIC 512 μg/mL, whereas **Poly-2c** is found to be the most effective in the series with MIC 256 μg/mL.

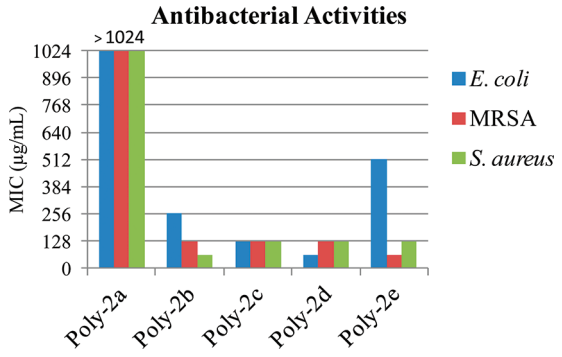

Figure 1. Determination of MIC values for the guanidinium polycarbodiimides.

Samples of **Poly-2b** obtained from a click reaction of **Poly-1b** of different molecular weight (i.e., M_n = 31000 and PDI = 3.74 vs M_n = 14000 and PDI = 2.19) showed similar activities.

As shown in the Figure 1, the polymers with shorter alkyl side chains, **Poly-2(b–d)** and **Poly-2e**, showed significant antibacterial activity against *S. aureus*, MRSA, and *E. coli*. The lower MIC of these polymers compared to the small molecule counterpart, guanidinium hydrochloride, which is reported to be MIC > 500 μg/mL for both *E. coli* and *S. aureus*[23] seems to be an advantage of these macromolecular systems. In addition, it is noteworthy that the relative concentration of guanidinium moiety in the repeat units in these polymers is much less (e.g., about one-third in **Poly-2c** repeat units) than that of guanidinium hydrochloride itself. In contrast, **Poly-2a** with the long dodecyl aliphatic tail and cationic guanidinium head at every repeat was found to be less effective compared to its small molecule counterpart, guanidinium hydrochloride. We postulate that this diminished activity in **Poly-2a** is due to shielding of the cationic headgroup by long aliphatic tail. Although, different polymers with a wide range of MIC against variety of bacteria have been reported,[2] the inevitable role of polymer backbone, microstructure, and architecture on bioactivity prevents us from direct comparison of bioactivities of these guanidinium functionalized water-soluble polycarbodiimides (**Poly-2(b–e)**) with other reported polymer systems. Antibacterial activities exhibited by polymers in this study appears to be moderate when comparison is made with some other polycations as their MIC range from 3.8 to >2000 μg/mL.[24] They do, however, have interesting hemoreactivity (vide infra) that in combination with these antibacterial properties make them bandage candidate materials for treating traumatic wounds.

Helical polycarbodiimides are intrinsically chiral and the fact that they can be prepared in predominant right (*P*-) or left (*M*-) screw senses[14,17] allowed us to explore the effect of chirality on these biotic activities. Both of the *P*- and *M*-handed polymers; **Poly-2c**(*-P*) and **Poly-2c**(*-M*) were obtained from **Poly-1c**(*-P*) and **Poly-1c**(*-M*) polymerized under the same polymerization conditions by using enantiomeric, *R*- and *S*-titanium-BINOL catalysts[25] with comparable molecular weights; resulting polymer from *R*- form of catalyst has M_n = 1200 and PDI = 3.22 and that from *S*- form of the catalyst has M_n = 1400 Da, PDI = 2.60. When tested for antibacterial activity, these two polymers, differing in the helical handedness, showed similar activities. The chirality of the helix did not seem to influence the antibacterial activity, indicating a nonspecific interaction of polymers with bacterial cell wall. This finding in our polymer system is in agreement with helical handedness not being influential in antimicrobial potency of peptides.[26,27]

To test hemoreactivity, we then performed the red blood cell hemolysis assay of the polymers on mechanically difibrinated sheep blood. The polymer with relatively long alkyl side chains, **Poly-2a**, was relatively less-hemoreactive with HD_{50} (concentration required for lysis of 50% red blood cells, RBCs) of 6.18 mMol. **Poly-2(b–e)** showed instant hemoreactivity. The RBCs precipitation occurred within a minute in the tested concentration range (3000–100 μM, Figure 2) for **Poly-2(b–e)**. The instant interaction between the polymers and the RBCs might be due to cross-linking between the cells mediated by cationic polymer surface resulting electrostatic complex. This later property of **Poly-2(b–e)** to induce instant RBCs precipitation without significant hemolysis needs further study to explore the potential use of these polymers in biomedical device applications.

Several cationic macromolecules such as polymer hydrogels,[28] poly(L-lysine),[29] and polyethylenimine (PEI)[30] have been reported to induce agglutination of blood cells, sometimes with high cationic charge densities causing higher cytotoxic effects.[31] PEGylation of cationic polymers is sometimes a

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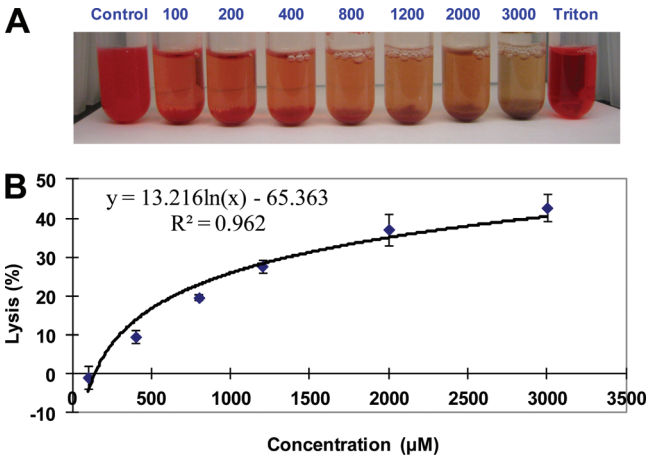

Figure 2. Hemoreactivity in the guanidinium polycarbodiimides: (A) an example of blood precipitation in **Poly-2(b–e)** and (B) hemolysis in **Poly-2a**.

strategy to mask the reactivity of cationic sequences in polymers for optimum activity.[32,33] In some other cases, macromolecules which accelerate blood clotting have been further investigated as promising candidates for hemostatic application in trauma and injuries.[28,34,35]

Unlike **Poly-2(b–e)** that caused RBCs precipitation, **Poly-2a** exhibited entirely different behavior toward RBCs with little hemolysis but without any precipitation. The diminished activities of **Poly-2a** with long aliphatic tail in repeat unit might have resulted from its local to global hydrophobic balance in the polymer. The long hydrophobic aliphatic tails conceal the cationic pendants and might have prevented them from significant interaction with cell surface. The mechanism of biological action of these polymers (**Poly-2(a–e)**) has not been delineated at this stage and needs further study. It is believed that positively charged guanidinium derivatives in macromolecules are attracted toward the negatively charged bacterial cell surface, resulting in the disruption of the cell wall and loss of membrane function causing bacterial cell death.[8,36]

In conclusion, we have developed new synthetic polymer-conjugates based on polycarbodiimides. Tuning of the polymer side chains and incorporation of bioactive guanidinium pendant groups resulted into cationic, amphiphilic water-soluble polycarbodiimides. Antibacterial activity of the guanidinium functionalized polymers against broad spectrum bacteria including drug-resistant MRSA showed that polycarbodiimide conjugates may be investigated as potential polymeric antibacterial agents/disinfectants. Precipitation of RBCs but without significant hemolysis caused by these polymers prevents the potential use of these polymers in systematic administration but opens an opportunity to explore antibacterial blood clotting polymers for surface applications such as polymer coatings or wound protection. Further optimization in the polymer system to develop better therapeutic materials is in progress in our laboratories and will be reported in due time.

■ ASSOCIATED CONTENT

Supporting Information

Further experimental details and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: bruce.novak@utdallas.edu.

Notes

The authors declare no competing financial interest.

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