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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Antimicrobial Metallopolymers and Their Bioconjugates with Conventional Antibiotics against Multidrug-Resistant Bacteria

Jiuyang Zhang,[†] Yung Pin Chen,[‡] Kristen P. Miller,[‡] Mitra S. Ganewatta,[†] Marpe Bam,[§] Yi Yan,[†] Mitzi Nagarkatti,[§] Alan W. Decho,[‡] and Chuanbing Tang*[,†]

[†]Department of Chemistry and Biochemistry, and [‡]Department of Environmental Health Sciences, University of South Carolina, Columbia, South Carolina 29208, United States

[§]Department of Pathology, Microbiology and Immunology, University of South Carolina, School of Medicine, Columbia, South Carolina 29209, United States

Supporting Information

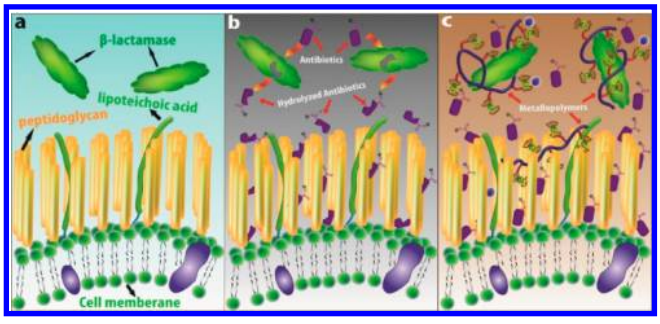
ABSTRACT: Bacteria are now becoming more resistant to most conventional antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA), a complex of multidrug-resistant Gram-positive bacterial strains, has proven especially problematic in both hospital and community settings by deactivating conventional β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems, through various mechanisms, resulting in increased mortality rates and hospitalization costs. Here we introduce a class of charged metallopolymers that exhibit synergistic effects against MRSA by efficiently inhibiting activity of β-lactamase and effectively lysing bacterial cells. Various conventional β-lactam antibiotics, including penicillin-G, amoxicillin, ampicillin, and cefazolin, are protected from β-lactamase hydrolysis via the formation of unique ion-pairs between their carboxylate anions and cationic cobaltocenium moieties. These discoveries could provide a new pathway for designing macromolecular scaffolds to regenerate vitality of conventional antibiotics to kill multidrug-resistant bacteria and superbugs.

Bacteria are rapidly developing resistance to one or more of the most frequently used antibiotics.[1] The extent of this global crisis is highlighted by the fact that, for example, annually two million patients suffer from hospital-acquired infections in the United States, with 99,000 deaths. Nearly 30% of these infections are identified as methicillin-resistant *Staphylococcus aureus* (MRSA).[1b,2] Today, 40−60% of *Staphylococcus aureus* strains in hospitals (hospital-associated MRSA, HA-MRSA) are resistant to penicillin, methicillin, and many other β-lactam antibiotics.[3] There are multiple mechanisms used by MRSA to undermine the effects of conventional antibiotics. For example, the class A β-lactamase enzyme catastrophically hydrolyzes conventional β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems.[4]

Currently, vancomycin and amoxicillin/clavulanic acid are among the most commonly used antibiotics for the treatment of MRSA infections.[1a,4a] Although these antibiotics are among the strongest of their classes, high-frequency use has resulted in their decreased activity against MRSA.[4a,b] In an effort to circumvent antibiotic resistance, novel β-lactamase inhibitors, including boronic acid derivatives, phosphonates, and phosphonamidates,[5] have been designed, although none of these agents have entered phase I development.[4b] Alternatively, synthetic macromolecules have been introduced as antimicrobial agents.[6] Rather than targeting penicillin-binding proteins as most β-lactam antibiotics do, cationic polymers or peptides[7] can disrupt thick cell walls or membranes and have shown efficacy against MRSA.[8] Some conventional antibiotics have exhibited activity against MRSA by their modification with polymers via covalent bonds or encapsulation in a polymeric matrix.[9] However, most of these strategies have been restricted by their inherent limitations, such as the high toxicity of cationic polymers and peptides, poor release of antibiotics, and relatively low targeting efficiency toward bacteria.[8a,9b] In contrast, organometallic compounds and macromolecules[10] have been previously used as anticancer drugs, targeting agents, and enzyme inhibitors.[11] However, their use as antimicrobial materials still remains in the early stages, and most have not yet achieved an optimal balance between toxicity and bioactivity.[11b,12]

Here we introduce a class of charged metallopolymers, which not only show high efficacy in reducing β-lactamase activity but also effectively lyse bacterial cells (Scheme 1). Our results

Scheme 1. Illustration of Several Key Interactions Involving β-Lactamase and β-Lactam Antibiotics: (a) MRSA Cells; (b) Typical β-Lactamase Hydrolysis of β-Lactam Antibiotics; and (c) Proposed Interactions between β-Lactam Antibiotic−Metallopolymer Bioconjugates, β-Lactamase, and Cell Wall



Received: February 2, 2014
Published: March 17, 2014

   dx.doi.org/10.1021/ja5011338 | *J. Am. Chem. Soc.* 2014, 136, 4873−4876

reveal that these metallopolymers attack both β-lactamase enzymes and cell walls and protect conjugated antibiotics via ion-pairing between polymers and antibiotics (Scheme 1). Specifically, these charged metallopolymers are based on cationic cobaltocenium-containing polymers. Due to the unique ability of cationic cobaltocenium moieties to complex with carboxylate anions, various commercial β-lactam antibiotics, including penicillin-G, amoxicillin, ampicillin, and cefazolin, can be protected from β-lactamase via the formation of stable ion-pairs with cationic cobaltocenium-containing polymers. Considering these synergistic attributes, these metallopolymers show high efficiency against multidrug-resistant MRSA, while exhibiting non-hemolytic activity and minimal *in vitro* and *in vivo* toxicity.

We and other groups recently discovered a class of cationic metallopolymers containing cobaltocenium moieties that exhibited unique ion-pairing ability.[13] Hexafluorophosphate (PF_6^-)-paired cobaltocenium-containing polymer, poly(2-(methacrylolyoxy)ethyl cobaltoceniumcarboxylate hexafluorophosphate) (M_n = 15,600 g/mol, M_w/M_n = 1.25), was prepared and used for this study.[13a,c] Halide anion (Cl^-, Br^-, and I^-)-paired cationic cobaltocenium polymers were subsequently prepared.[13c,d] All halide-paired polymers are hydrophilic and highly soluble in water (solubility >800 mg/mL).

β-Lactamase production and excretion is a major defense mechanism employed by several drug-resistant bacterial pathogens, such as various strains of MRSA.[4a,b] Effects of cationic cobaltocenium-containing polymers on β-lactamase activity were conducted by incubation of β-lactamase [obtained from HA-MRSA (ATCC 29213) extracellular solution] with nitrocefin and halide-paired cobaltocenium-containing polymers together. Nitrocefin is a chromogenic cephalosporin that is classified as a β-lactam antibiotic and is typically used to indicate the existence of β-lactamase.[14] A solution of nitrocefin with a pristine β-lactam ring typically appears yellow with an absorption peak near 380 nm. However, after hydrolysis of the β-lactam ring by β-lactamase, the solution typically turns red with an absorption peak near 480 nm (the control sample).[14] As shown in Figure 1a,c, incubation of β-lactamase with halide-paired polymers and nitrocefin resulted in little hydrolysis (solutions remained yellow with low absorption at 482 nm), although those with PF_6^--paired polymers resulted in significant hydrolysis of nitrocefin (the solution turned red with high absorption at 482 nm). A time-dependent study was conducted by measuring absorption at 482 nm at different time intervals (Figure 1b). The inhibition of β-lactamase is shown in Figure 1d. More than 80% inhibition of β-lactamase activity was achieved by metallopolymers at 5 μM. The use of metallopolymers at 10 μM shut down the β-lactamase hydrolysis completely (Figure S10). However, negligible inhibition of β-lactamase was observed for PF_6^--paired metallopolymer, possibly due to its poor solubility in water.

The protection of nitrocefin by metallopolymers may be due to the unique ability of cationic cobaltocenium moieties to form ion-pairs with the carboxylate anion in nitrocefin. Carboxylate anions in nitrocefin could readily perform counterion-exchange with halide counterions in these cobaltocenium-containing polymers, leading to the formation of nitrocefin–metallopolymer conjugates with 1:1 pairing between nitrocefin and cobaltocenium moieties (Figure 2a,b and Figure S1). As illustrated in Figure S7, preliminary studies indicated that such ion-pairing interaction may block the electrostatic anchoring by amino acid residue (Lys_{234})[15] and could also prevent the key

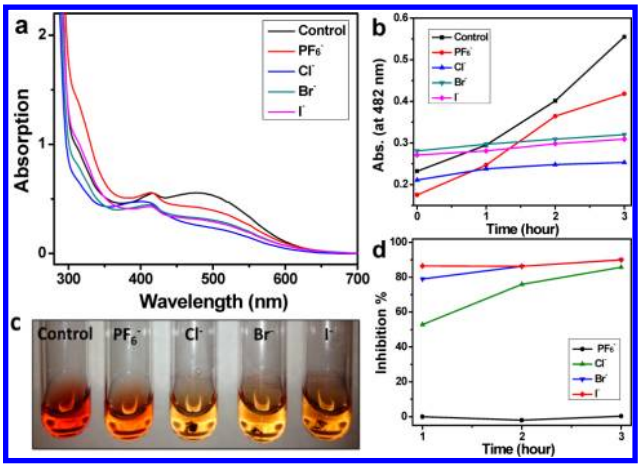


Figure 1. (a) UV–vis absorption of nitrocefin solution with 5 μM anion-paired metallopolymers and β-lactamase incubated for 3 h. (b) Incubation time-dependent absorption (at 482 nm) of nitrocefin solution with different anion-paired metallopolymers at 5 μM and β-lactamase. (c) Optical view of nitrocefin solution with different anion-paired metallopolymers at 5 μM and β-lactamase incubated for 3 h. (d) Level of β-lactamase activity inhibition by different anion-paired cationic cobaltocenium-containing polymers at 5 μM.

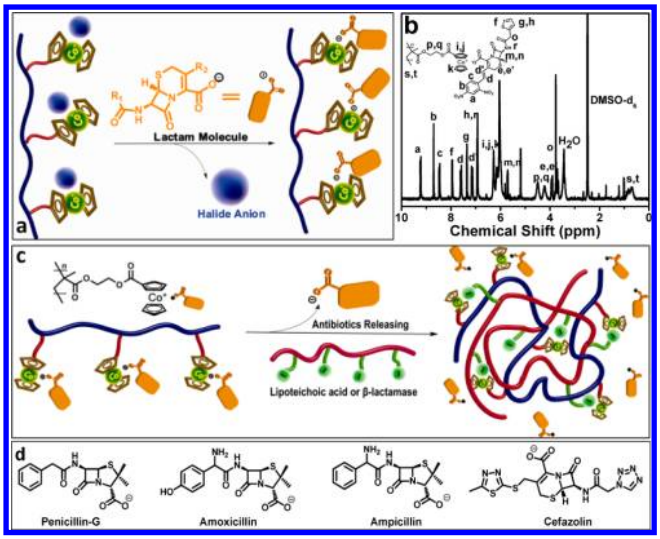


Figure 2. (a) Formation of ion-pairs between β-lactam antibiotics and cationic cobaltocenium-containing polymers. (b) ^{1}H NMR spectrum for ion-pairs of nitrocefin and cationic cobaltocenium-containing polymers. (c) Antibiotic release from antibiotic–metallopolymer ion-pairs via lipoteichoic acid or β-lactamases. (d) Four β-lactam antibiotics used in this study.

acylation and deacylation steps from Glu_{166} in β-lactamases produced by MRSA.[4a,b] However, detailed mechanisms are still under study.

Considering the similarity in structure between nitrocefin and other β-lactam antibiotics, cationic cobaltocenium-containing polymers could be extended to protect other conventional β-lactam antibiotics using a similar approach (Figure 2a,d). As indicated by ^{1}H NMR analysis (Figures S2–S5), penicillin-G, cefazolin, amoxicillin, and ampicillin can form conjugates with cationic metallopolymers. Furthermore, we found that antibiotics were released when antibiotic–metallopolymer bioconjugates performed ion-exchange with negatively charged cell walls or with carboxylate anions in extracellular solution (Figure

dx.doi.org/10.1021/ja5011338 | J. Am. Chem. Soc. 2014, 136, 4873–4876

2c). We carried out model studies to mimic interactions between cationic cobaltocenium-containing polymers and cell walls, as well as β-lactamase. Lipoteichoic acid was chosen as it critically contributes to the negative charge of cell wall (Scheme 1).[16] We also selected poly(acrylic acid), as its pK_a (~4.2) is very similar to that of Glu_{166} in β-lactamase scaffold. These interactions are included in Figures S8 and S9. Interestingly, the results showed that the ion-pair interactions can enable lipoteichoic acid or β-lactamase to bind with these metallopolymers and subsequently release previously complexed β-lactam antibiotics (Figure 2c).

Disk-diffusion assays were used to evaluate the antimicrobial activities of penicillin-G, cefazolin, amoxicillin, ampicillin, and their bioconjugates with cobaltocenium-containing polymers against drug-resistant MRSA cells (Figure 3a and Figure S11).

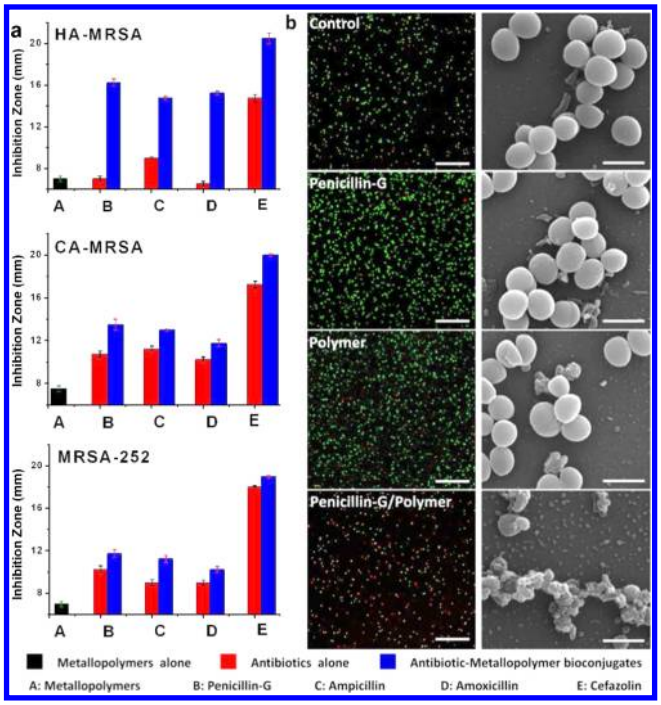


Figure 3. (a) Results of disk-diffusion assays to test antimicrobial effects of conventional antibiotics (red bar), Cl^--paired metallopolymers (black bar), and their conjugates (blue bar) against HA-MRSA, CA-MRSA, and MRSA-252 (concentrations of metallopolymer were 1−2.2 μM, exact concentrations are summarized in Table S2). (b) CSLM images (left column) and corresponding SEM images (right column) of HA-MRSA cells incubated respectively in the presence of control solution, 5.6 μM penicillin-G (2 μg/mL), 1 μM Cl^--paired cationic cobaltocenium-containing polymers (12.5 μg/mL), and penicillin-G−metallopolymer bioconjugate (5.6 μM penicillin-G and 1 μM metallopolymers). CSLM imaging employed BacLight live/dead stain (green indicates live cells, red indicates dead cells). Scale bars in confocal images, 50 μm; scale bar in SEM images, 1 μm.

Three different strains of MRSA, community-associated MRSA (CA-MRSA, ATCC 1717), HA-MRSA (ATCC 29213), and MRSA-252 (ATCC 1720), were incubated for 24 h with antibiotics only, Cl^--paired metallopolymers only, and a mixture of the two components (compositions are summarized in Table S2). For mixtures of two components, the molar ratio of cobaltocenium moieties to antibiotics was controlled at >1:1, recognizing that in extracellular solution, species with carboxylate groups would complex with cobaltocenium moieties. Cl^--metallopolymers and antibiotics were mixed to form stable antibiotic−metallopolymer ion-pairs before being added to the disks. As shown in Figure 3a and Figure S11, at a concentration of 1−2.2 μM, metallopolymers alone showed very little inhibition against MRSA cells, while most antibiotics alone also exhibited low toxicity. However, their corresponding bioconjugates showed significantly enhanced effects in activities against different strains of MRSA, especially for HA-MRSA. The growth of CA-MRSA and MRSA-252 was also inhibited, but to a lesser extent than HA-MRSA, which may be due to other resistant mechanisms in CA-MRSA and MRSA-252.[1c] The quantitative difference of inhibition level between antibiotics only and their bioconjugates is summarized in Table S3. The inhibition of HA-MRSA growth was also observed by confocal scanning laser microscopy (CSLM) and scanning electron microscopy (SEM) imaging, as shown in Figure 3b.

We further discovered that these cationic metallopolymers themselves also showed efficient inhibition against different MRSA cells when their concentrations were increased to about 5 μM (3−5 times of their concentrations in the above antibiotic−metallopolymer bioconjugate study). This is different from other types of β-lactamase inhibitors, as they are mostly non-antimicrobial. The inhibitory concentration (IC_{90}) of each halide-paired cobalt-containing polymer was determined for methicillin-sensitive *Staphylococcus aureus* (MSSA, ATCC-1718), HA-MRSA, CA-MRSA, and MRSA-252. As shown in Figure 4a, both Br^-- and Cl^--paired metallopolymers

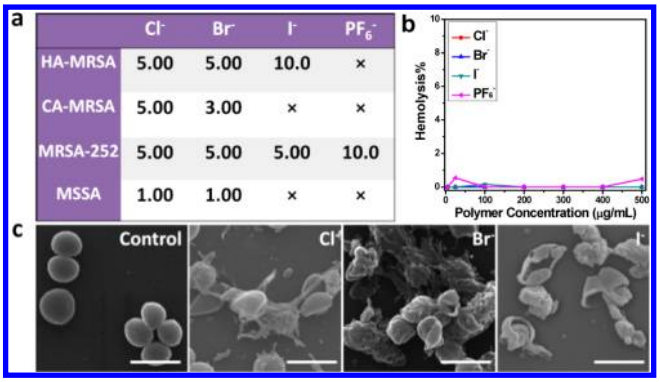


Figure 4. (a) IC_{90} values (μM) of Cl^--, Br^--, I^--, and PF_6^--paired cobaltocenium-containing polymers against MSSA, HA-MRSA, CA-MRSA, and MRSA-252 from standard solution microbroth measurement. × indicates less than 90% inhibition at 10 μM (Supporting Information, Table S1). For halide- and PF_6^--paired cobaltocenium-containing polymers, 1 μM is in the range of 12.5−15.6 μg/mL, depending on the counterion used. (b) Hemolytic activities of halide-paired cationic cobaltocenium-containing polymers against mouse red blood cells. (c) SEM images of HA-MRSA cells before (control) and after incubation with halide-paired cobaltocenium-containing polymers at 5 μM for 9 h. Scale bars represent 1 μm.

have IC_{90} values at 3−5 μM against HA-MRSA, CA-MRSA, and MRSA-252, while only 1.00 μM against MSSA cells. I^--paired metallopolymers are slightly less effective, while PF_6^--paired cobaltocenium-containing polymers showed much weaker antimicrobial effects, mostly due to their limited solubility in aqueous media.[13d] Compared with other cationic antimicrobial polymers, these metallopolymers showed comparable or even lower minimum inhibitory concentrations (15−100 μg/mL) against bacteria.[8,9c] SEM was utilized to illustrate the influence of halide-paired cationic cobaltocenium-containing polymers on MRSA cell membranes. Figure 4c shows representative images

dx.doi.org/10.1021/ja5011338 | J. Am. Chem. Soc. 2014, 136, 4873−4876

of HA-MRSA cells treated with halide-paired cationic metallopolymers. Compared to control cells, all MRSA cells exhibited partial or complete membrane lysis. Through electrostatic interactions, cationic cobaltocenium-containing polymers could adsorb to the negatively charged MRSA cell walls, damage the cell walls, and thus lead to cell death, similar to other cationic antimicrobial polymers.[6b,8]

Although these cationic metallopolymers exhibited excellent abilities to lyse microbial cells, they showed negligible hemolytic effects on red blood cells. As shown in Figure 4b, halide-paired metallopolymers showed extremely low levels of hemolysis (less than 1%) at concentrations up to 500 μg/mL for metallopolymers (~40 μM for Cl^--paired cobaltocenium-containing polymers). This result is important because it indicates our metallopolymers exhibit an extremely low cytotoxicity to red blood cells and a high selectivity against bacterial cells.[8a,9c] Furthermore, cytotoxicity of these metallopolymers was also investigated in *in vitro and in vivo* tests, which indicated little toxicity[8] (see discussion in Supporting Information, Figures S12–S14).

In conclusion, a class of metallopolymers, cobaltocenium-containing polymers, was discovered to form bioconjugates with various β-lactam antibiotics, including penicillin, ampicillin, amoxicillin, and cefazolin, via ionic complexation. These antibiotic–metallopolymer bioconjugates showed high resistance toward β-lactamase-assisted hydrolysis of β-lactam antibiotics and significantly improved efficacy against various strains of MRSA cells over conventional antibiotics. In addition, these metallopolymers themselves, at higher concentrations, also showed excellent antimicrobial activities against different strains of MRSA by selectively disrupting their cell membranes, while maintaining extremely low cytotoxicity against red blood cells and low *in vivo* toxicity. These discoveries could pave a new platform to design antibiotics and antimicrobial agents to battle multidrug-resistant bacteria and superbugs.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

tang4@mailbox.sc.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Support from the National Science Foundation (CHE-1151479 and DMR-1206072) is acknowledged.

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