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Trio Act of Boronolectin with Antibiotic-Metal Complexed Macromolecules toward Broad-Spectrum Antimicrobial Efficacy

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Abstract

Bacterial infections, particularly by Gram-negative pathogens, have become a serious threat to global healthcare due to the diminishing effectiveness of existing antibiotics. We report a nontraditional therapy to combine three components in one macromolecular system, in which boronic acid adheres to peptidoglycan or lipopolysaccharide via boron-polyol based boronolectin chemistry, cationic metal polymer frameworks interact with negatively charged cell membranes, and β -lactam antibiotics are reinstated with enhanced vitality to attack bacteria via evading the detrimental enzyme-catalyzed hydrolysis. These macromolecular systems exhibited high efficacy in combating pathogenic bacteria, especially Gram-negative strains, due to synergistic effects of multicomponents on interactions with bacterial cells. *In vitro* and *in vivo* cytotoxicity and hemolysis evaluation indicated that these multifunctional copolymers did not induce cell death by apoptosis, as well as did not alter the phenotypes of immune cells and did not show observable toxic effect on red blood cells.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00132. Experimental details; NMR spectra, TGA thermograms, and FTIR spectra of monomers and copolymers; minimum inhibitory concentration (MIC) evaluations of PCo-PPB copolymers and PCo homopolymer against *S. aureus* and *E. coli*; antimicrobial effect of polymer conjugates and penicillin-G against Gram-positive bacteria using disk-diffusion assays; 3-D plots of results from disk-diffusion assays; CLSM images and FESEM images of Gram-positive bacteria (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Discoveries of new antibiotics have significantly decreased since the 1980s.¹ The situations become even worse when resistance to one antibiotic can spread to others within the same categories.^{2–4} Concurrently, efforts for antibiotic discovery by the pharmaceutical industry have exhibited an astonishing decline, the majority of which have primarily focused on modifications of existing classes of antibiotics. While these efforts continue, the escalating crisis of antibiotic resistance has made it imperative to develop a new portfolio of innovative antimicrobial therapies.^{5,6}

Unlike Gram-positive bacteria, Gram-negative pathogens possess double membranes with a variety of additional resistance mechanisms to evade antibiotics.⁷ It is a multifaceted challenge for drugs to successfully penetrate both barriers and kill bacteria. In order to generate and better tailor new biochemical properties for drug discovery, we need to understand and overcome barriers for antimicrobial agents targeting Gram-negative bacteria. Concurrently, we ought to evaluate and validate alternative, nontraditional therapies for the treatment of bacterial infections.

Antimicrobial peptides can bind their cationic amino sequences to the negative bacterial surface followed by insertion of hydrophobic groups into the membrane to combat drug-resistant bacteria.^{8–11} However, peptides could suffer low stability and high manufacturing cost. Cationic polymers with quaternary ammonium or phosphonium groups, as

antimicrobial peptide mimics, have recently garnered a great deal of attention, which are easily prepared and more difficult for bacterial cells to launch resistance.^{12–24} However, many of these antimicrobial agents are toxic toward mammalian cells due to nonselective damage to cell morphology, thus limiting their roles in clinical settings. In addition, most of these therapeutics have targeted Gram-positive bacteria. Therefore, restoring the vitality of conventional antibiotics and developing potent antimicrobial agents that are nontoxic to mammalian cells are essential for treating bacterial infections.^{1,5}

On the other hand, lectins are a class of carbohydrate-binding proteins that are highly specific for sugar moieties.²⁵ Boronic acid chemistry, which is based on the fast formation of boronate esters between boronic acid and polyol-containing molecular targets, has been widely utilized for glucose and glycoprotein sensing, drug/DNA delivery, cell adhesion, etc.^{26–32} Boron–polyol interactions in synthetic macromolecules, in many scenarios, are of fundamental importance to human health, plant growth, and quorum sensing among bacteria. Such unique interactions are utilized for the design of synthetic lectins, termed “boronlectins”, which are particularly appealing when boronic acids interact with carbohydrates such as sugars in bacterial cell components.^{25,33}

Herein, we report a trio act of nontraditional therapy combining boronlectin, antibiotics, and metal-containing macromolecules in nullifying multiple strains of bacteria with high antimicrobial efficacy. As shown in Scheme 1A, a cationic metal-containing macromolecular framework binds to the outer membrane via electrostatic interactions; boronlectin chemistry is utilized to bind with various sugars on lipopolysaccharides (LPS) and peptidoglycan, respectively, from Gram-negative and Gram-positive bacteria. A bioconjugate between metal-containing macromolecules and conventional β -lactam antibiotics could circumvent major enzymatic hydrolysis via β -lactamase(s) and thus resume the effectiveness of antibiotics attacking peptidoglycan which is critical for cell wall synthesis. Together, these multiple killing components provide multifaceted and versatile means for penetrating cellular barriers to fight bacterial resistance. The current work is based on our early discovery that cationic cobaltocenium-containing metallopolymers could lyse different strains of Gram-positive strains.³⁴ However, these polymers are not as potent against Gram-negative strains of bacteria, in part, due to their limited interactions with bacterial cell membranes.

RESULTS AND DISCUSSION

Preparation of Multi-Component Macromolecules

We integrated cationic cobaltocenium, boronic acid, and antibiotics together in a single macromolecule (Scheme 1B). Specifically, we prepared poly(cobaltocenium–phenylboronic acid) (PCo-PPB) copolymers, which were then installed with penicillin via ionic complexation. Cobaltocenium, a cationic and high-oxidation state form of cobaltocene, has been demonstrated with excellent chemical stability and superior binding ability to large anions.^{35–43} The PCo-PPB copolymers were synthesized via reversible-addition–fragmentation chain transfer (RAFT) polymerization using 2-cobaltocenium amidoethyl methacrylate hexafluorophosphate (CoAEMAPF₆) and 3-acrylamidophenylboronic acid (APBA) as comonomers. The details of RAFT polymerization are provided in the

Supporting Information. The PCo-PPB copolymers are hydrophilic and highly soluble in water after ion-exchange from PF_6^- to Cl^- .⁴⁴

We synthesized three PCo-PPB copolymers with different fractions of phenylboronic acid by changing molar ratios of CoAEMAPF₆ to APBA (Table S1), while keeping their molecular weight similar (~14 500 g/mol). The proportion of APBA in the copolymers was ~20 wt % (PCo-PPB-1), 15 wt % (PCo-PPB-2), and 8% (PCo-PPB-3), respectively (Figure S2a, thermogravimetric analysis). In addition to characteristic peaks from NMR spectra (Figure S1), Fourier-transform infrared (FTIR) spectra of PCo-PPB copolymers exhibited a stretching vibrational peak of B–OH at 1350 cm^{-1} from APPA, carbonyl stretching of ester bands at 1720 cm^{-1} , and C–O stretching from CoAEMAPF₆ at 1120 cm^{-1} , which further supported a successful synthesis of the copolymers (Figure S2b). With the increase of phenylboronic acid fractions in copolymers, the intensity of the B–O peak increased, while the intensity of the ester weakened.

We then mixed penicillin-G with PCo-PPB copolymers to form a bioconjugate (labeled as PCo-PPB-Peni) via ionic complexation between cationic cobaltocenium and anionic antibiotic. The level of antibiotic loading could be tuned by simply changing the ratio of penicillin to cobaltocenium.

Boronolectin: Interactions with Sugars To Enhance Adhesion with Bacterial Cells

Boronolectin is centered on boron–polyol interactions (Scheme 2). There are a variety of polyols in the sugars of critical components of Gram-negative bacteria. Lipopolysaccharide (LPS) is located in the outer membrane of Gram-negative bacteria, consisting of a lipid, a polysaccharide composed of O-antigen, and outer core and inner core joined by covalent bonds. When antimicrobial agents diffuse toward the microbial cell surface, they must first cross LPS and the outer membrane. The O-antigen is attached to the core oligosaccharide and comprises the outermost domain of the LPS molecule.⁴⁵ Given the similarity of sugar contents in the Gram-negative bacteria, we carried out studies on the interactions between PCo-PPB copolymers and model sugars including L-rhamnose, D-glucose, and D-mannose (Scheme 2). Compared with FTIR spectra of PCo-PPB copolymer and model sugar alone, the characteristic peaks of boronate ester (B–O–C stretching vibration) at 1050 cm^{-1} were found in all spectra of PCo-PPB-sugar conjugates (Figure 1A–C), which suggested that phenylboronic acid in the copolymers can form boronate ester with either cis-1,2-diol or cis-1,3-diol in any of these representative sugars.⁴⁶

In Gram-positive bacteria, antimicrobial agents must first penetrate peptidoglycan before reaching the plasma membrane. Peptidoglycan is a polymer consisting of sugars and amino acids that form a mesh-like layer outside the plasma membrane of most bacteria, forming the cell wall. The peptidoglycan layer is substantially thicker in Gram-positive bacteria than in Gram-negative bacteria. In order to verify the interactions between PCo-PPB copolymers and Gram-positive bacteria, the peptidoglycan extracted from *Staphylococcus aureus* (*S. aureus*) was used as the model biomacromolecule. Similarly, the characteristic peak of boronate ester appeared in the spectrum of PCo-PPB-peptidoglycan conjugates (Figure 1D), which suggested that peptidoglycan was successfully bounded with the copolymer via the formation of boronate esters between boronic acids from copolymers and polyols from

peptidoglycan. Such interactions may be an important factor in dictating antimicrobial efficacy of PCo-PPB copolymers.

Antimicrobial Activities of Multi-Component Metal-Iopolymers

Initially, we tested the antimicrobial activities of PCo-PPB copolymers against Gram-negative *E. coli* using disk-diffusion assays. As shown in Figure 2A, when compared with a homopolymer of CoAEMA having chloride as the anion (PCo, $M_n = 15\,000$ g/mol) and polyphenylboronic acid (PPB, $M_n = 15\,500$ g/mol), PCo-PPB copolymers showed significantly enhanced activities (PCo-PPB-3 as an example). The bacteria were incubated with different concentrations of polymers for 6 h, and then, the OD₆₀₀ values of microbial cultures were measured. The PCo-PPB copolymers exhibited higher antibacterial activities than the PCo homopolymer. The minimum inhibitory concentrations (MICs) of PCo-PPB significantly decreased, in comparison to PCo homopolymer. Given the three copolymers we tested, the results suggested that the antimicrobial efficacy did not exhibit a clear dependence on the fraction of PPB (with PPB in the range of 8–20 wt %) (Figure 2B). As for Gram-positive bacterium *S. aureus*, the PCo-PPB copolymers showed higher antimicrobial activities than *E. coli* (Figure S3). Although the MICs of PCo-PPB decreased to 40–70 $\mu\text{g/mL}$, in comparison to >100 $\mu\text{g/mL}$ for PCo homopolymer alone (Figure S4), these polymers did not have superior antimicrobial activities.

We then installed PCo-PPB copolymers with β -lactam antibiotics, for which we chose penicillin-G. As shown in Scheme 1, we mixed penicillin-G with a copolymer PCo-PPB-1 to form a bioconjugate (PCo-PPB-Peni) via strong electrostatic interaction between cationic cobaltocenium (metal cation) and anionic antibiotic (carboxylate group). High antibiotic loading capacity (31 wt % with a molar ratio of cobaltocenium to penicillin-G of $\sim 1:0.6$) could be easily obtained. Disk-diffusion assays were first used to evaluate antimicrobial activities of PCo-PPB-Peni bioconjugates against four strains of Gram-negative bacteria (*E. coli*, *P. vulgaris*, *K. pneumonia*, *P. aeruginosa*). To better compare bactericidal efficiency, we prepared a bioconjugate containing only PCo and penicillin-G (PCo-Peni, with 43 wt % penicillin), but without phenylboronic acid, as a control. As shown in Figure 2C, when the amount of penicillin-G was 5 μg , penicillin-G alone exhibited very low antimicrobial efficacy against *E. coli*, and the inhibition zone was almost negligible (~ 6 mm). In contrast, PCo-Peni and PCo-PPB-Peni bioconjugates displayed a significant enhancement, and inhibition zones increased to 8 and 12 mm, respectively, with the same amount of penicillin-G (5 μg). By increasing the amount of penicillin-G to 10 μg , the inhibition zones of penicillin-G, PCo-Peni, and PCo-PPB-Peni appreciably increased to 9, 16, and 22 mm, respectively. When tested against other three bacteria (*P. aeruginosa*, *P. vulgaris*, *K. pneumonia*) (Figure 2D–F), once again, PCo-PPB-Peni bioconjugates exhibited the strongest antimicrobial activities at different amounts of penicillin-G. Similarly, as for Gram-positive strains *S. aureus* and *E. faecalis*, PCo-PPB-Peni conjugate also showed enhanced activities compared to PCo-Peni and penicillin alone (Figures S5 and S6).

The MICs of penicillin-G, PCo-Peni, and PCo-PPB-Peni against different Gram-negative strains are given in Table 1. It should be mentioned that the MIC values are based on the effective concentration of penicillin in the polymer–penicillin conjugates. The MICs of PCo-

PPB-Peni against Gram-positive strains are $3.7 \mu\text{g/mL}^{-1}$ for *E. coli*. These values are significantly lower than those of PCo-Peni ($7.1 \mu\text{g/mL}^{-1}$) and penicillin ($12.6 \mu\text{g/mL}^{-1}$). Similarly, as for the other three Gram-negative strains, MIC values of PCo-PPB-Peni conjugate are almost two times less than PCo-Peni and three times less than penicillin alone. All these bioconjugates have MICs substantially lower than copolymers without antibiotics.

The inhibition effect by PCo-PPB-Peni on four different Gram-negative bacterial strains was further confirmed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Results of LIVE/DEAD bacterial viability assays using CLSM suggested penicillin-G or PCo-PPB copolymers alone resulted in relatively few dead bacterial cells at relatively low concentrations (Figure 3). In contrast, almost all bacteria incubated with PCo-PPB-Peni were killed. From SEM images, we can observe that PCo-PPB-Peni damaged bacterial membranes, made bacteria cells shrink, and effectively killed them, while the untreated bacteria (control groups) exhibited a typical (healthy) spherical or rod morphology with a smooth surface. The antimicrobial activity of PCo-PPB-Peni conjugates against Gram-positive bacteria *S. aureus* and *E. faecalis* was also evaluated by CLSM and SEM (Figures S7 and S8).

We postulated that the high bactericidal efficacy of PCo-PPB-Peni bioconjugates may result from synergistic effects, which were centered on the building blocks of cobaltocenium and phenylboronic acid. In one scenario, the phenylboronic acid group can attach to bacteria through binding of polymers with LPS on the cell surface. Alternatively, the cationic cobaltocenium can not only interact with the negatively charged bacterial membrane but additionally block the electrostatic chelation between the β -lactam antibiotic and amino acid residue of β -lactamase. This would prevent hydrolyses of penicillin-G by bacterial β -lactamases. The effect of PCo-PPB copolymers on the β -lactamase activity was conducted by UV-visible spectra using nitrocefin as an indicator (Figure 4). In the control, the addition of β -lactamase resulted in a rapid color change (from yellow to red) in the nitrocefin solution, and a characteristic absorption peak appeared near 480 nm due to hydrolysis of the β -lactam ring in nitrocefin. However, when PCo-PPB copolymers were first bound with nitrocefin for the formation of conjugates, the solution color remained yellow with a very low absorption at 480 nm after the addition of β -lactamase, suggesting that PCo-PPB can inhibit β -lactamase activity and prevent hydrolysis of β -lactam ring in the antibiotic.

Cytotoxicity against Mammalian Cells

In order to determine the toxicity of PCo-PPB copolymers, we performed both *in vitro* and *in vivo* experiments testing their ability to induce programmed cell death known as apoptosis, as well as necrosis in immune cells. For this purpose, the cells were cultured with PBS (vehicle control), 10 or 50 $\mu\text{g/mL}$ of copolymers for 24 h, and then stained with fluorochrome-labeled Annexin V-propidium iodide (PI). Annexin V has high affinity for phosphatidylserine (PS) and can detect the translocation of membrane PS from the inner face of the plasma membrane to the cell surface, which is a first step in apoptosis induction and PI stains necrotic cells. Double-positive cells represent late apoptotic cells. Naive immune cells upon culture in tissue culture medium undergo cell death in a fraction of the cells as shown by positivity for staining in early apoptotic (Annexin V+, PI-), late apoptotic

(Annexin V+, PI +), and necrotic (Annexin V–, PI+) cells in the PBS-treated negative control group. We found that the copolymer-treated splenocytes were susceptible to induction of cell death to the same extent as PBS-treated cells (Figure 5A). Similarly, following *in vivo* treatment of mice with PBS or copolymers (10 mg/kg body weight) for 48 h, we observed that the percentages of apoptotic and necrotic splenocytes from copolymer-injected and PBS-injected mice were comparable, indicating an extremely low cytotoxic nature of PCo-PPB copolymers to immune cells (Figure 5B).

Next, we phenotyped immune cells for detection of cell subpopulations by targeting unique markers with a specific antibody followed by detection using flow cytometry, which is a very sensitive technique for quantitative determination of large numbers of cells. We stained the cells fluorochrome-labeled CD3, CD4, CD8, and CD19 specific antibodies and employed flow cytometry to detect whether the PCo-PPB copolymers influenced the different populations of T and B cell lineages in the splenocytes after intraperitoneal injection of copolymers for 48 h. We observed that the treatment of mice with these copolymers did not alter the percentages of the immune cells when compared to PBS-treated groups. The percentages of all cell types, including CD3+ T cells, CD3+CD4+ T helper/regulatory cells, and the CD3+CD8+ cytotoxic T cells as well as the CD19+ B cells from mice injected with the copolymers, were similar to those of the PBS-injected mice (Figure 5C). These data again strongly suggested that the copolymers did not alter the ratio of immune cells.

We analyzed the toxicity of PCo-PPB copolymers on murine red blood cells (RBCs) by evaluating whether they could lead to hemolysis of RBCs. We found that, even at a concentration of the copolymers as high as 500 $\mu\text{g/mL}$, lysis of RBCs was extremely low (<10%) when compared to the negative control group (Figure 5D).

In summary, we developed a nontraditional therapy of antimicrobial agents that combine a trio act of boronolactin, cationic metallopolymer, and antibiotics. The newly formed phenylboronic acid-containing penicillin-complexed cobaltocenium metallopolymer exhibited robust, synergistic antibacterial activities through the electrostatic absorption to negatively charged bacterial membranes/cell walls via the cationic cobaltocenium moiety and the binding of phenylboronic acid to peptidoglycan or lipopolysaccharides on the bacterial cells, as well as reinstatement of the vitality of antibiotics, and exhibited excellent antibacterial activity against a variety of strains of both Gram-negative and Gram-positive bacteria. This novel platform of multifunctional macromolecules could open a new avenue toward sustainable approaches to attacking Gram-negative bacteria and addressing potential bacterial resistance.

METHODS

Synthesis of Poly(cobaltocenium-phenylboronic acid) (PCo-PPB) Copolymers

Cobaltocenium monomer, 2-cobaltoceniumamidoethyl methacrylate hexafluorophosphate (CoAEMAPF₆), was synthesized according to a reported method.⁴⁷ PCo-PPB copolymers were synthesized via RAFT polymerization:^{38,48} CoAEMAPF₆ (490 mg, 1.0 mmol), APBA (95 mg, 0.5 mmol), DTPA (4.15 mg, 0.01 mmol), and AIBN (0.5 mg, 0.003 mmol) were

dissolved in a 1.0 mL solution of DMF/H₂O (95/5) in a 10 mL Schlenk flask and then degassed by purging N₂ for 30 min. The mixture was then irradiated at 70 °C for 12 h. After reaction, the mixture was precipitated in cold dichloromethane for three times and vacuum-dried. Finally, the PCo-PPB copolymers became soluble in water through a facile phase-transfer ion-exchange from PF₆⁻ to Cl⁻ using TBACl according to a previous report.⁴⁴ A typical procedure was as follows: 1 mL of PF₆⁻ paired PCo-PPB copolymer (30 mg/mL in acetonitrile) was slowly dropped into a 5 mL TBACl solution (in acetonitrile) under vigorous stirring. After stirring for 5 min, the precipitated Cl⁻ paired PCo-PPB copolymer was collected and washed by acetonitrile three times to remove any remaining PF₆⁻ anions and excess TBACl. The solid copolymer was then vacuum-dried and collected.

Synthesis of PCo-PPB-Peni Bioconjugates and Antibiotic Release Experiments

PCo-PPB copolymer (10 mg) and antibiotic penicillin-G sodium salt (10 mg) were dissolved in deionized water (1 mL) with molar ratios (penicillin salt to cobaltocenium moieties) of 1.1 to 1. The solution was stirred for 12 h and then dialyzed against 3 L of deionized water for 12 h. The solution in a dialysis bag was collected and freeze-dried. The PCo-PPB-Peni bioconjugate was obtained. The penicillin-G mass loaded into PCo-PPB-Peni bioconjugate was calculated by subtracting the mass of penicillin-G in the total dialysate solution from the total mass of the drug in the initial solution measured by a UV visible spectrophotometer at 250 nm and a standard penicillin calibration curve.

Evaluation of Antimicrobial Effects

The following bacterial strains were purchased from ATCC: *Staphylococcus aureus* (*S. aureus*, ATCC-33591), *Escherichia coli* (*E. coli*, ATCC-11775), *Klebsiella pneumoniae* (*K. pneumoniae*, ATCC-35596), *Proteus vulgaris* (*P. vulgaris*, ATCC-33420), *Enterococcus faecalis* (*E. faecalis*, ATCC-19433), and *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC-10145). For these bacteria, a single colony was inoculated in 30 mL of Tryptic Soy broth (TSB) at 37 °C for 24 h, shaking at 190 rpm/min. All bacteria were grown to an optical density of about 1.00 (OD₆₀₀ = 1.00) for further use.

To conduct the agar disk-diffusion assays, actively growing cultures of each bacterial strain on Mannitol salt agar (MSA) were inoculated on TSB agar plates. The bacterial growth culture (cell concentrations were 1.0 × 10⁶ CFU/mL; 10 μL) was diluted to 1 mL in TSB, and 100 μL of that was spread on TSB agar plates to form a bacterial lawn covering the plate surface. Then, 6 mm (diameter) filter discs were added to the plate surface. PCo-PPB copolymers or PCo-PPB-Peni bioconjugates at different amounts in 30 μL of water were added to disks, and the plates were incubated at 28 °C for 18 h. The development of a clear zone around the disk was indicative of the ability of agents to kill bacteria.

The minimum inhibitory concentrations (MIC) of PCo-PPB copolymers and PCo homopolymers were determined. 50 μL of aqueous solution of PCo-PPB copolymers or PCo homopolymers with different concentrations was added to 96-well plates. Then, 150 μL of bacterial TSB solution (OD₆₀₀ = 1.00) was added to the wells. The bacterial TSB solution without polymers was used as the control. The assay plate was incubated at 37 °C for 12 h. Bacterial growth was detected at OD₆₀₀ and was compared to controls of bacterial TSB

solution without polymers. All assays were carried out in duplicate in the same assay plate. Inhibitory percentages were calculated by following equation.

$$\text{inhibition\%} = \frac{OD_{600}(t) - OD_{600}(0)}{OD_{600}(t)_c - OD_{600}(0)_c}$$

Here, $OD_{600}(0)$ indicates the initial OD_{600} value and $OD_{600}(t)$ is the OD_{600} value for bacteria after incubation with polymers for t hours. $OD_{600}(0)_c$ is the initial OD_{600} value and $OD_{600}(t)_c$ is the OD_{600} value for control samples after incubation for t hours.

LIVE/DEAD Bacterial Viability Assays

The six bacteria strains were inoculated and prepared by a similar procedure used for inhibitory concentration determination studies. One mL of active bacterial stock of bacteria strains was introduced to 5 μg of penicillin-G, 11 μg of PCo-PPB copolymer, and 16 μg of PCo-PPB-Peni bioconjugates (penicillin-G weight: 5 μg), respectively. An untreated cell suspension was used as the control. Following an 18 h incubation at 37 °C, 1 μL of LIVE/DEAD BacLight (Bacterial Viability Kit; Invitrogen Inc.) was added to the incubation solution. After incubation for 15 min, cells were imaged using a Leica TCS SP5 CLSM with a 63 $\times$ oil immersion lens. When excited at 488 nm with argon and helium/neon lasers, bacteria with intact membranes display green fluorescence (emission = 500 nm) and bacteria with disrupted membranes fluoresce red (emission = 635 nm).

Bacterial Morphology Assays

The morphologies of different bacteria after incubation of PCo-PPB-Peni bioconjugates were examined by FE-SEM with a similar procedure. In general, 10 μL of bacteria cell solution was grown on one glass slide in a 12-well plate containing 1 mL of TSB medium at 37 °C overnight. Cell suspensions were diluted to $OD_{600} = 1.0$. 16 μg PCo-PPB-Peni bioconjugates (penicillin-G weight: 5 μg) was added to the 1 mL cell stock solution, respectively and incubated at 37 °C overnight. A cell suspension without any chemicals was used as the control. The samples were then fixed in cacodylate buffered with 2.5% glutaraldehyde solution (pH = 7.2) for 2–3 h at 4 °C and postfixes with 1% osmium tetroxide at 4 °C for 1 h. The samples were dried under a critical point, then coated with gold using a Denton Des II Sputter Coater for 120 s, and observed by FE-SEM. An untreated cell suspension was used as the control.

Effects of PCo-PPB Copolymers on β -Lactamase Activity

50 μL of nitrocefin (DMSO solution 1.0 mg/mL) and PCo-PPB copolymers with different amounts (100, 200, and 400 μg) were added into 1 mL of H_2O and stirred for 12 h. Then, 1 μL of β -lactamase PBS buffer solution (0.1 mg/mL, determined by Biorad Protein Assay) was added to the above solution. A solution without copolymer was used as a control. The β -lactamase activity was measured by UV-vis absorbance at 482 nm after 1 h.

Mice and Splenocyte Preparation

Normal C57BL/6 female mice (6–8 weeks old) were procured from the Jackson Laboratory (Bar Harbor, ME) and used when mice were about 12 weeks old. Animals were housed at the University of South Carolina Animal facility. All necessary care and maintenance of the animals were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*.

Spleens from mice were collected and placed in complete RPMI 1640 medium. Immediately, single-cell suspensions of splenocytes were prepared and RBC lysed as described previously.⁴⁹ Cell viability was determined on a hemocytometer (Hausser Scientific, Horsham, PA) by trypan blue dye staining of the cells and using an inverted phase-contrast microscope (Nikon, Inc., Melville, NY).

In Vitro Splenocyte Culture with the Copolymers

Splenocytes (1×10^6 CFU/mL) were cultured with PBS or PCo-PPB copolymers at either 10 or 50 μ g/mL for 24 h in complete RPMI 1640 medium (10% heat-inactivated fetal bovine serum, 10 mM L-glutamine, 10 mM HEPES, and 100 μ g/mL penicillin/streptomycin) at 37 °C and 5% CO₂.

In Vivo Exposure of Mice to the Copolymers

To determine the effect of the PCo-PPB copolymers on the splenocytes in normal mice, a single dose of 10 mg/kg body weight was administered (intraperitoneal injection) into groups of mice. Mice treated with PBS were used as control.

Detection of Apoptosis in Splenocytes

Splenocytes from mice exposed to PCo-PPB copolymer (10 mg/kg body weight) or PBS were analyzed for apoptosis by using the FITC Annexin V apoptosis detection kit with propidium iodide (PI) (Biolegend, CA, USA) following instructions from the manufacturer. In brief, splenocytes (1×10^6 CFU/mL) from various groups of mice were cultured for 24 h in complete RPMI 1640 medium, after which, the cells were washed twice with PBS, followed by washing twice with cell staining buffer provided with the kit. Washed cells were resuspended in Annexin V binding buffer, and FITC conjugated Annexin V was added, followed by PI. The cells were gently vortexed and incubated at RT in the dark for 15 min and immediately analyzed in a flow cytometer.

Annexin V is a member of the annexin family of intracellular proteins. These proteins bind to phosphatidylserine (PS) in a calcium-dependent manner. Detection of Annexin V to determine apoptosis is a straightforward and very sensitive technique. Under normal conditions, PS is found only on the intracellular leaflet of the plasma membrane in healthy cells. As membrane asymmetry is lost during early apoptosis, PS translocate to the external leaflet. Fluorochrome-labeled Annexin V can then be employed to target and identify apoptotic cells specifically. However, Annexin V binding alone cannot differentiate between apoptotic and necrotic cells. Thus, to distinguish the necrotic from apoptotic cells, propidium iodide solution (PI) is used in combination. PI will be excluded by early apoptotic

cells, while late stage apoptotic cells and necrotic cells will stain positively due to the passage of these dyes into the nucleus where they bind to DNA.

Assessment of Immune Cell Subpopulations

Splenocytes (1×10^6) from PBS- or copolymer-treated groups of mice were washed with PBS (Invitrogen) and incubated in the dark for 30 min on ice with 0.5 μg of the following anti-mouse primary monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)–conjugated anti-CD3, phycoerythrin (PE)–anti-CD8, allophycocyanin (APC)–anti-CD4 (all from Biolegend, CA, USA), or FITC–anti-CD19 (BD Pharmingen, San Diego, CA, USA). For triple-staining studies, directly conjugated monoclonal antibodies were simultaneously added to the sample. Negative controls consisted of cells that were not stained with mAbs and represented autofluorescence. Flow cytometric analysis was performed by a Cytomics FC500 flow cytometer (Beckman Coulter, Inc. USA) using the operating software. At least a hundred thousand cells were collected per sample. Dead cells, clumps, and debris were excluded electronically by gating on forward (FSC) versus side scatter (SSC).

Hemolysis Evaluation

Blood was collected from mice in heparinized tubes and diluted by mixing 800 μL of blood with 1000 μL of PBS. Copolymer samples were prepared in PBS at concentrations of 10, 50, 100, and 500 $\mu\text{g}/\text{mL}$, and 60 μL of the diluted blood samples was added to 3 mL of copolymers, PBS, or 0.1% Triton-X100 in PBS. The samples were incubated for 1 h at 37 °C followed by centrifugation for 10 min at 1500 rpm. Supernatants were collected, and OD was measured at 545 nm to calculate hemolysis rate by using the equation, $\text{HR} = (\text{AS} - \text{AN})/(\text{AP} - \text{AN})$, where AS, AN, and AP are OD values of the supernatants from test samples, negative control (PBS), and positive control (0.1% Triton-X100)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

FTIR spectra of (A) L-rhamnose, PCo-PPB copolymer, and PCo-PPB-Rhamnose conjugate; (B) D-mannose, PCo-PPB copolymer, and PCo-PPB-Mannose conjugate; (C) D-glucose, PCo-PPB copolymer, and PCo-PPB-Glucose conjugate; (D) peptidoglycan, PCo-PPB copolymer, and PCo-PPB-peptidoglycan conjugate. Note: the B–O–C is stretching vibration ($1030\text{--}1050\text{ cm}^{-1}$) of boronate ester. The PCo-PPB copolymer (PCo-PPB-1, 10.0 mg) and model sugars (10 mg) were mixed in water (2 mL, pH = 7.0) for 6 h at room temperature and then examined by FTIR after freeze-drying.

Figure 2.

Agar diffusion tests: (A, B) against *E. coli* by PCo, PPB, PCo-PPB-1, PCo-PPB-2, and PCo-PPB-3; (C–F) against *E. coli*, *P. aeruginosa*, *P. vulgaris*, and *K. pneumoniae* by penicillin-G, PCo-Peni, and PCo-PPB-Peni. All compounds at different amounts in 30 μ L of water were added to disks, and the plates were incubated at 28 $^{\circ}$ C for 18 h.

Figure 3.

CLSM and SEM images of control, PCo-PPB (11 $\mu\text{g/mL}$), penicillin-G (5 $\mu\text{g/mL}$), and PCo-PPB-Peni (16 $\mu\text{g/mL}$, with the concentration of penicillin-G at 5 $\mu\text{g/mL}$) against four strains of bacteria. All scale bars are 2.0 μm (using BacLight live/dead stain, green indicates live cells, red indicates dead cells). Bacteria concentrations were 1.0×10^6 CFU/mL.

Figure 4.

UV-vis absorption of nitrocefin solution with PCo-PPB copolymers (PCo-PPB-1 as an example) after adding β -lactamase for 1 h. 50 μL of nitrocefin (DMSO solution 1.0 mg/mL) and PCo-PPB copolymers with different amounts (100, 200, and 400 μg) were added into 1 mL of H_2O and stirred for 12 h. Then, 1 μL of β -lactamase PBS buffer solution (0.1 mg/mL, determined by Biorad Protein Assay) was added to the above solution. A characteristic absorption peak appeared near 480 nm due to hydrolysis of the β -lactam ring in nitrocefin.

Figure 5.

Cytotoxicity of copolymers on mammalian cells: (A) Flow cytometry of murine splenocytes and (B) the percentage of apoptotic cells after injecting with PBS or copolymers at the concentration of 10 mg/kg body weight *in vivo*. Splenocytes were collected after 48 h, cultured for 24 h, and followed by detection of apoptosis using flow cytometry after staining of Annexin V. (C) Splenocytes from *in vivo*-treated mice were stained with anti-CD3, -CD4, -CD8, and -CD19 Abs and analyzed by flow cytometry. (D) Hemolysis was detected by culturing RBCs with copolymers and determining hemolysis. PBS was used as the control.

Scheme 1.

(A) Illustration of Synergistic Antimicrobial Mechanisms of Multifunctional Macromolecules against Gram-Negative Bacteria via a Trio Act of Boronolactin Chemistry on Lipopolysaccharide, Electrostatic Attraction between Cationic Metal and Negatively Charged Membrane, and Reinstated Antibiotic Targeting on Peptidoglycan; (B) Preparation of Multi-Component Macromolecules (PCo-PPB and PCo-PPB-Peni) Containing Boronic Acid, Cobaltocenium, and/or Penicillin via RAFT Polymerization and Ion-Exchange

Scheme 2.

Boronolactin Chemistry Involving Phenylboronic Acid Polymers and Polyols and Representative Sugars Containing Cis-1,2 and 1,3-Diols from LPS and/or Peptidoglycan

Table 1

MICs of Different Antimicrobial Agents against Gram-Negative Strains

antimicrobial agents	minimum inhibitory concentration ($\mu\text{g/mL}$) (MIC based on the concentration of penicillin)			
	<i>E. coli</i>	<i>P. vulgaris</i>	<i>P. aeruginosa</i>	<i>K. pneumonia</i>
penicillin	12.6	15.2	12.6	18.0
PCo-Peni	7.1	8.6	9.2	8.1
PCo-PPB-Peni	3.7	5.6	6.1	4.8