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Biodegradable polycaprolactone metallopolymer–antibiotic bioconjugates containing phenylboronic acid and cobaltocenium for antimicrobial application†

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Abstract

This paper reports antimicrobial metallopolymer containing biodegradable polycaprolactone as the backbone with boronic acid and cobaltocenium as the side chain. While boronic acid promotes interactions with bacterial cells *via* boronolactone with lipopolysaccharides, cationic cobaltocenium facilitates the unique complexation with anionic β -lactam antibiotics. The synergistic interactions in these metallopolymer–antibiotic bioconjugates were evidenced by re-sensitized efficacy of penicillin-G against four different Gram-negative bacteria (*E. coli*, *P. vulgaris*, *P. aeruginosa* and *K. pneumoniae*). The degradability of the polyester backbone was validated through tests under physiological pH (7.4) and acidic pH (5.5) or under enzymatic conditions. These metallopolymer exhibited time-dependent uptake and reduction of cobalt metals in different organs of mice *via in vivo* absorption, distribution, metabolism, and excretion (ADME) tests.

Introduction

The rise of antimicrobial resistance to traditional antibiotics has raised the alarm to the threat of uncontrollable healthcare crisis.^{1–3} Bacterial infections have become common in many fields, leading to significant healthcare cost.^{4–6} Each year, about 700 000 people are killed by bacterial infections all over the world, of which over 20% are caused by more resistant and virulent microorganisms, *i.e.*, “superbugs”.⁷ Compared with infections caused by Gram-positive bacteria, the infections by Gram-negative strains are associated with even higher morbidity and mortality.^{2,8,9} There is a strong need to develop new antimicrobial agents and revitalize old antibiotics.^{10,11}

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Conflicts of interest

The authors declare no competing financial interest.

Antimicrobial peptides (AMPs) are the first line of a host defense system with common features regardless of their biological origin.^{12–15} They are biopolymers, consisting of tens to hundreds of charged amino acid residues, which can interact with both hydrophobic and hydrophilic moieties.¹⁶ The hydrophilic (*e.g.*, cationic charges) structure can initiate nonspecific electrostatic interactions with negatively charged bacterial cell surfaces, concurrently with the insertion of hydrophobic motifs of AMPs into the nonpolar lipids of bacterial membranes.^{17,18} However, antimicrobial peptides have some intrinsic drawbacks, including high manufacturing costs and low lifetime stability.

Significant efforts have been diverted to develop AMP mimics: synthetic cationic macromolecules.^{19–24} A variety of synthetic polymers containing diverse cationic charges (*e.g.*, quaternary ammonium, phosphonium, imidazolium, pyridinium, and pyrrolidinium) with a delicate balance of amphiphilicity have been designed for antimicrobial applications.^{25–31} However, in contrast to AMPs, the importance of the degrade-ability of synthetic polymers is largely overlooked. A desirable antimicrobial polymer is expected to be released from biological systems after carrying out the therapeutic functions. However, except few antimicrobial polymers,^{32–34} most of them are not degradable,^{14,19,23,25,35,36} which may circumvent renal filtration and increase toxicity to patients.

We recently reported a class of noncytotoxic antimicrobial metallopolymer containing cationic cobaltocenium.^{36–41} Cobaltocenium, an oxidized form of neutral cobaltocene, is a much softer cation than conventional quaternary ammonium. According to Pearson's acid–base theory,⁴² cobaltocenium can form strong complexes with soft bases such as carboxylates. It was discovered that cobaltocenium-containing metallopolymer can form unique bioconjugates with conventional β -lactam antibiotics possessing anionic carboxylates,³⁸ such as penicillin, amoxicillin, and cefazolin, *via* ion complexation. These metallopolymer–antibiotic bioconjugates could efficiently kill Gram-positive multi-drug resistant (MDR) bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA).³⁷ By disabling β -lactamase enzymes, cobaltocenium metallopolymer re-sensitize β -lactam antibiotics to kill bacteria. We further conceptualized boron-polyol based boronolactin chemistry into cobaltocenium metallopolymer to kill Gram-negative bacteria by binding phenylboronic acid to lipopolysaccharides on the bacterial cells.^{38,43,44} However, all these metallopolymer are based on (meth)acrylic polymer backbones, which are well known to be non-degradable.

Herein we report a new methodology to develop biodegradable polycaprolactone-based metallopolymer that have a polyester as the backbone (Scheme 1). Polycaprolactone (PCL) is a type of biodegradable polyester, which has been FDA-approved for many biomedical applications.⁴⁵ Phenylboronic acid and cobaltocenium motifs were grafted onto the side chain of PCL. After binding with penicillin-G, the metallopolymer–antibiotic bioconjugates retained the vitality of antibiotics and exhibited excellent antibacterial activity against four different strains of Gram-negative bacteria. We carried out *in vivo* absorption, distribution, metabolism, and excretion (ADME) tests and demonstrated that PCL copolymers possessed time-dependent degradability.

Results and discussion

Synthesis of metallopolymer–antibiotic bioconjugates

Caprolactone copolymers containing phenylboronic acid and cobaltocenium, PCL-PPB-PCo, were synthesized *via* a combination of ring-opening polymerization (ROP) and click chemistry (Scheme 1). Firstly, the preparation of azide-substituted polycaprolactone (PCL-N₃) followed the early work reported by us.³³ The click reaction was then carried out between PCL-N₃ and 3-(2-propynylaminocarbonyl)phenylboronic acid pinacol ester with only a fraction of azide involved, yielding phenylboronic acid-substituted PCL (PCL-PPB-N₃). Then, PCL-PPB-PCo copolymers were finally synthesized *via* click reaction between PCL-PPB-N₃ and ethynyl cobaltocenium hexafluorophosphate. The PCL-PPB-PCo copolymers were hydrophilic and highly soluble in water after deprotection of phenylboronic acid pinacol ester and phase-transfer ion-exchange of counterion from PF₆[−] to Cl[−].

Chemical structures of PCL-Cl, PCL-N₃, PCL-PPB-N₃ and PCL-PPB-PCo were characterized with the aid of ¹H NMR and Fourier-transform infrared spectroscopy (FTIR). According to ¹H NMR spectra shown in Fig. 1a, the proton next to azide group (labeled with “a”) shifted from 4.2 to 3.8 ppm, following the azide replacement of chlorine group. The integration of ¹H NMR spectra indicated that all chlorine groups were converted to the azide groups, which was also confirmed by the appearance of characteristic strong absorption of azide groups at ~2120 cm^{−1} in FTIR spectra (Fig. 1b). After the click reaction between PCL-N₃ and 3-(2-propynylaminocarbonyl)phenylboronic acid pinacol as well as ethynyl cobaltocenium, chemical shift at 7.7–8.4 ppm was assigned to the characteristic aromatic protons from phenylboronic acid pinacol ester. The peaks at 5.50–6.25 ppm corresponding to the cyclopentadienyl protons (C_p) of cobaltocenium were clearly present in the spectra of PCL-PPB-PCo copolymers, indicating the successful grafting of both moieties to the PCL side chain. FTIR spectra further confirmed the quantitative click reaction as the residue azide peak at ~2120 cm^{−1} remained after the first click reaction and completely disappeared after the second click reaction. Meanwhile, some new absorption bands at ~1650, 1050 and 800 cm^{−1} emerged, corresponding to the absorption of triazole, boronic ester and cyclopentadienyl groups, respectively. Due to the strong electrostatic interaction between cationic cobaltocenium moieties and the stationary phase of microstyragel columns, it was very challenging to obtain the GPC signal of cobaltocenium-containing copolymers. The molecular weight of PCL-PPB-PCo copolymers and molar fractions of PPB and PCo were determined by ¹H NMR spectroscopy (Fig. S1 and S2 in ESI[†]).

In search for the optimal compositions of PCL-PPB-PCo copolymers that would perform high efficacy of antimicrobial activity, we synthesized nine PCL-PPB-PCo copolymers with different molecular weights and molar percentages of phenylboronic acid (Table 1). The initial antimicrobial studies were carried out using the standard disk-diffusion assay following the Kirby Bauer disk diffusion test,⁴⁶ and *E. coli* (ATCC-11775) was chosen as a representative of Gram-negative bacteria. Firstly, the influence of molecular weight of

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PCL-PPB-PCo on the antimicrobial activity was studied. Keeping the molar percentage of phenylboronic acid at 20%, the molecular weight of PCL-PPB-PCo was varied from 4500 to 35 000 g mol⁻¹ (denoted from PCL-PPB-PCo-1 to PCL-PPB-PCo-5). Fig. S3a in the ESI† suggested that the higher antimicrobial activity was achieved using a molecular weight of 15 000 g mol⁻¹. Next, using this optimal molecular weight, the effect of molar percentages of phenylboronic acid on antimicrobial activity was performed. PCL-PPB-PCo copolymers with 10%, 15%, 20%, and 30% PPB were synthesized (denoted from PCL-PPB-PCo-6 to PCL-PPB-PCo-9). In addition, 100% PPB grafted PCL (PCL-PPB) and 100% PCo grafted PCL (PCL-PCo) polymers were also prepared to compare their antimicrobial ability. As shown in Fig. S3b,† compared with PCL-PCo and PCL-PPB, the PCL-PPB-PCo copolymers showed significantly enhanced antimicrobial activities, the highest antimicrobial efficacy was achieved with the copolymer containing 15% molar percentage of phenylboronic acid (PCL-PPB-PCo-7). Copper (Cu) as a heavy metal can induce metal-catalyzed oxidation reactions that may damage proteins, membranes, or DNA, showing potent antimicrobial activities.⁴⁷ To assess the potential antibacterial effect of CuI remained in PCL-PPB-PCo copolymers after click reaction, we first tested the amount of CuI in copolymers (~8 µg mg⁻¹) *via* inductively coupled plasma mass spectrometry (ICP-MS). Then, we compared the antimicrobial abilities of PCL-PPB-PCo copolymers (PCL-PPB-PCo-1 to PCL-PPB-PCo-5, 250 µg) and CuI compound (2 µg), keeping the same amount of CuI. As shown in Fig. S4,† all PCL-PPB-PCo copolymers showed high antibacterial activity against both *E. coli* and *P. aeruginosa*, while the same amount of CuI exhibited negligible antibacterial effect. This suggested the antibacterial activity of copolymers came from the synergistic action between boronic acid and cationic cobaltocenium,³⁸ rather than trace remaining CuI.

Antimicrobial assay of copolymers and bioconjugates

To improve the antimicrobial efficacy, PCL-PPB-PCo copolymers were conjugated with a β -lactam antibiotic, penicillin-G (Fig. 2a). The copolymer PCL-PPB-PCo-7 (M_n = 15 000 g mol⁻¹, 15 mol% PPB and 85 mol% PCo) was chosen to complex with penicillin-G *via* the unique ionic complexation between cationic cobaltocenium and anionic antibiotic, yielding a bioconjugate labeled as **PCL-PPB-PCo-Peni**. High antibiotic loading capacity (38 wt%, the molar ratio of cobaltocenium moiety to penicillin-G is ~1.3 : 1) was obtained (see TGA data in Fig. S5,†). Upon adding the PCL-PPB-PCo-Peni bioconjugates into bacterial culture medium, the cationic cobaltocenium would interact with negatively charged bacterial membranes. The PCL-PPB-PCo-Peni bioconjugates can release the antibiotics with the aid of polyvalent effect. Meanwhile, the phenylboronic acid group can bind with lipopolysaccharides on bacterial outer leaflets through the formation of boronic esters, facilitating intracellular uptake of the bioconjugates (Fig. 2b).³⁸ Disk-diffusion assays were first used to evaluate antimicrobial activities of PCL-PCo-PPB-Peni bioconjugates against four strains of Gram-negative bacteria (*E. coli*, *P. vulgaris*, *P. aeruginosa* and *K. pneumoniae*) using inhibition zones in diameter (mm). A bioconjugate containing 100% PCo functionalized PCL with penicillin-G and without phenylboronic acid (PCL-PCo-Peni) was chosen as a control in the disk diffusion assay. The amount of penicillin-G was maintained constant in all samples. As shown in Fig. 2c, in contrast to penicillin-G (19 mm), PCL-PCo-Peni (22 mm) and PCL-PPB-PCo-Peni (24 mm) bioconjugates with the same amount of penicillin-G (15 µg) displayed a significant enhancement against *E. coli* with

increased inhibition zones. By increasing the amount of penicillin-G to 20 μg , the inhibition zones of penicillin-G, PCL-PCo-Peni, and PCL-PPB-PCo-Peni appreciably increased to 21 mm, 24 mm, and 26 mm, respectively. When tested against other three additional bacteria, the PCL-PPB-PCo-Peni bioconjugates also outperformed against penicillin-G. To quantify the inhibition efficacy of PCL-PPB-PCo-Peni conjugates, bacteria were incubated with different antimicrobial agents in tryptic soy broth (TSB) solution for 8 h, and bacterial growth was detected with OD_{600} values (Fig. 2d). Compared with the control groups, TSB solutions of these bacteria without antimicrobial agents, the OD_{600} value of PCL-PPB-PCo-Peni conjugates for *E. coli* was only 0.05, approximately 32 times lower than that of the control (1.60), 8 times lower than penicillin-G (0.40), and 4 times lower than PCL-PCo-Peni (0.2), respectively. All other three bacteria showed lower viability after treatment with PCL-PPB-PCo-Peni conjugates, compared with the groups treated by PCL-PCo-Peni conjugates and penicillin-G alone, which was consistent with the results from disk diffusion assays.

The minimum inhibitory concentration (MIC) values of PCL-PPB-PCo-Peni conjugates, PCL-PCo-Peni conjugates and penicillin-G alone against these four Gram-negative bacteria were summarized in Table 2. All MIC values of copolymer–penicillin bioconjugates were reported based on the effective penicillin-G concentration in their conjugates. In the case of *E. coli*, the MIC value of PCL-PPB-PCo-Peni conjugates was about $4.5 \mu\text{g mL}^{-1}$, which was much lower than the values of penicillin-G ($14.8 \mu\text{g mL}^{-1}$) and PCL-PCo-Peni conjugates ($7.6 \mu\text{g mL}^{-1}$). Similarly, MIC values of PCL-PCo-Peni conjugates against the other three Gram-negative bacteria were almost 3 times or 1.5 times lower than those of penicillin-G and PCL-PCo-Peni conjugates, respectively.

The inhibition effects by PCL-PPB-PCo-Peni bioconjugates on the four bacterial strains were further confirmed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). LIVE/DEAD bacterial viability assay was performed and analyzed by CLSM. As shown in Fig. 3a, when bacterial cells were exposed to PCL-PPB-PCo-Peni conjugates, more cell death and lower cell density were observed, as shown by the red or yellow stain. In contrast, cells in control groups were stained green, revealing the most bacterial cells alive. From SEM images in Fig. 3b, the PCL-PPB-PCo-Peni bioconjugates showed the ability to damage and kill bacteria with abnormal cell morphologies. The untreated bacteria groups (control groups) exhibited the typical spherical or rod morphologies with smoother surfaces.

Finally, the hemolysis of PCL-PPB-PCo-7 copolymers against red blood cells (RBCs) was performed by evaluating whether they could lead to lysis of RBCs. Even at a higher concentration of PCL-PPB-PCo-7 copolymers, $500 \mu\text{g mL}^{-1}$, lysis of RBCs was extremely low ($<10\%$) when compared to the negative control group (Fig. S6[†]). Thus, these studies further demonstrated that PCL-PPB-PCo-7 copolymers could show a minimum toxicity to RBCs. Cytotoxicity of PCL-PPB-PCo-7 was further performed using human primary umbilical vein endothelial cells (HUVEC) cultured with vascular cell basal medium containing endothelial cells growth factors (ATCC, Manassas, VA). The PCL-PPB-PCo-7 copolymers with different concentrations were added to the cell culture medium when cells were at 70–80% confluency. As shown in Fig. S7,† in comparison with the control (medium without copolymers), the PCL-PPB-PCo-7 copolymers only induced a negligible

cytotoxicity effect after 1, 8 and 24 h. Even at a high concentration of 500 $\mu\text{g mL}^{-1}$, the cytotoxic effect against HUVEC is less than 20%.

Degradation of copolymers

The degradability of PCL-PPB-PCo-7 copolymers was tested according to three different methods: acid-catalyzed degradation, hydrolytic degradation, and enzymatic degradation. The acidic degradation was carried out in a diluted HCl/H₂O solution ($\sim 0.15\text{ M HCl}$). PCL-PPB-PCo copolymers (20 mg) were added into HCl/H₂O (5 mL) in a dialysis bag (Fisher Science, seamless cellulose dialysis tubing, cutoff molecular weight 1000), which was placed into 100 mL HCl/H₂O solution (Fig. 4a). Next, 1 mL dialysate was taken out at different time to check the UV absorption of cobaltocenium at 270 nm (Fig. S8†). Using a previously published method,⁴⁸ according to the UV data, the PCL-PPB-PCo copolymers showed about 70% degradation after 3 days in the acidic condition (Fig. 4a).

The sites of bacterial infection are typically acidic, with the pH approximately 5.5, because of the presence of organic acids (such as acetic and lactic acids) produced by bacteria and inflammation.⁴⁹ Hence, the hydrolytic degradation experiment of PCL-PPB-PCo copolymers was conducted in buffer solutions with pH values at 7.4 (physiological condition) and 5.5 (infection condition). As shown in Fig. 4b, the degradation level of copolymers was only 10% in physiological condition (pH = 7.4) after 21 days. However, at pH = 5.5 (red curve in Fig. 4b), the degradation accelerated, and the amount of degraded copolymers reached 60% at 21 days, much higher than that in the physiological environment, suggesting that the PCL-PPB-PCo copolymers could degrade faster in an infection condition.

A time-dependent *in vitro* enzymatic degradation study of PCL-PPB-PCo copolymers was performed using *Pseudomonas* lipase (1 U mL^{-1}) as the enzyme in PBS solution (pH = 7.4) at 37 °C. Lipases were widely found in microorganisms, and lipase from *Pseudomonas* showed high catalytic activity towards the hydrolysis of PCL polymers.^{50,51} As shown in Fig. 4c, the PCL-PPB-PCo copolymers showed a fast degradation profile under the enzymatic condition. About 70% copolymers were degraded after 12 h. Then, the degradation rate decreased because the activities of the enzyme declined with time. After 3 days, the degradation level of copolymers reached 83%.

Absorption, distribution, metabolism, and excretion (ADME) of copolymers

To investigate the *in vivo* degradation of PCL-PPB-PCo copolymers, “absorption, distribution, metabolism, and excretion” (ADME) tests were carried out in mice.^{47,52} A single dose of PCL-PPB-PCo polymers (10 mg per kg body weight) was administered (intraperitoneal injection) into groups of mice. Mice treated with PBS were used as control. Major organs (liver, kidney, lung, and spleen), serum, and urine were collected from mice after injection of copolymers for 1 day and 7 days. The cobaltocenium concentrations in these organs were quantitatively analyzed by inductively coupled plasma mass spectrometry (ICP-MS). As shown in Fig. 5, compared with the control group, cobaltocenium concentrations in all organs increased after injection for 1 day. Especially in liver, cobaltocenium concentration was detected to be 750 ng g⁻¹, which was much

higher than that in the control group (33 ng g^{-1}). Cobaltocenium concentrations in urine increased from 60 ng g^{-1} to 300 ng g^{-1} at Day 1, which suggested that copolymers started to be excreted from the body during the first 24 h. After the injection of copolymers for 7 days, cobaltocenium concentrations in all organs, except kidney, reduced up to 70% of their original levels from Day 1. Meanwhile, cobaltocenium concentrations in urine also decreased (to about 60%), indicating the degradation was still in progress, and complete degradation may need longer time. It is worth noting that the cobaltocenium concentrations in serum remained low level after injection for 1 day and 7 days, similar to that in the control group, revealing that the cobaltocenium did not spread into or accumulate in blood/systemic circulation.

Conclusions

In summary, we prepared a class of biodegradable polycaprolactone-based metallopolymers containing phenylboronic acid and cobaltocenium. These copolymers were screened for antimicrobial efficacy to optimize the macromolecular compositions and molecular weight. Then, the selected copolymer PCL-PPB-PCo (15% PPB, 85% PCo, 15 kDa) was conjugated with penicillin-G, which synergistically enhanced antimicrobial effects. When tested against four different Gram-negative bacterial strains (*E. coli*, *P. aeruginosa*, *P. vulgaris*, and *K. pneumoniae*), the copolymer bioconjugates showed significantly better inhibition than penicillin-G alone. Further, *in vitro*, and *in vivo* evaluations demonstrated these copolymers have high biocompatibility, low cytotoxicity, and good degradability. The ADME assays indicated that the biodistribution of cobaltocenium (both uptake and removal) was time-dependent with significant reduction of cobalt in a longer time. This kind of nontoxic, degradable metallopolymers can be served as promising antimicrobial biomaterials and antibiotic sensitizers.

Methods

Synthesis of phenylboronic acid and cobaltocenium-containing polycaprolactone copolymers (PCL-PPB-PCo)

The azide substituted poly(ϵ -caprolactone) (**PCL-N₃**) (1.00 eq.), 3-(2-propynylaminocarbonyl)phenylboronic acid pinacol ester (0.20 eq.), and CuI (0.02 eq.) were mixed in a Schlenk flask. The mixture was purged with N₂ for 10 min. DBU (0.02 eq.) was dissolved in dry THF and transferred to the flask. The solution was stirred at 35 °C overnight to obtain phenylboronic acid pinacol ester substituted poly(ϵ -caprolactone) (**PCL-PPB-N₃**). After the click reaction, the obtained PCL-PPB-N₃ polymer was passed through a neutral aluminum oxide column to remove the copper catalyst and then precipitated in water three times to get the final product. In the second step, cobaltocenium-substituted copolymer (**PCL-PPB-PCo**) was synthesized *via* click reaction between PCL-PPB-N₃ and ethynyl cobaltocenium hexafluorophosphate. The final product was dried in a vacuum oven at room temperature. Finally, counterion of the obtained PCL-PCo-PPB copolymer was changed from PF₆⁻ anion to Cl⁻ anion by using tetrabutylammonium chloride (**TBACl**) through a facile phase-transfer ion-exchange reaction according to a previous report.⁵³ A typical procedure was as follows: 1 mL of PF₆⁻ paired PCL-PPB-PCo copolymer (30 mg mL^{-1} in

acetonitrile) was slowly added into 5 mL of TBACl solution (in acetonitrile) under vigorous stirring. After 5 minutes, the precipitated Cl^- paired PCL-PPB-PCo copolymer was collected and washed by acetonitrile three times to remove any remaining PF_6^- anions and excess TBACl. The purified copolymer was then vacuumdried and collected.

Synthesis of PCL-PPB-PCo-Peni bioconjugates

PCL-PPB-PCo copolymer (10.0 mg, 1.00 eq.) and penicillin-G sodium salt (10.0 mg, 1.10 eq.) were dissolved in deionized water (1 mL). The solution was stirred for 12 h and then dialyzed against 3 L of deionized water for 12 h. The solution in the dialysis bag was collected and freeze-dried to obtain PCL-PPB-PCo-Peni bioconjugate. The loaded penicillin-G mass in the PCL-PPB-PCo-Peni bioconjugate was calculated by a subtracting method. The mass of penicillin-G in the total dialysate solution subtracted from the total mass of the penicillin-G in the initial solution, which was measured by a UV visible spectrophotometer at 250 nm using a standard calibration curve of penicillin-G.

Evaluation of antimicrobial effects

The following bacterial strains were purchased from ATCC: *Klebsiella pneumoniae* (*K. pneumoniae*, ATCC-35596), *Escherichia coli* (*E. coli*, ATCC-11775), *Proteus vulgaris* (*P. vulgaris*, ATCC-33420), and *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC-10145). For these bacteria, a single colony was inoculated in 30 mL Tryptic Soy broth (TSB) at 37 °C for 24 h, shaking at 190 rpm. All bacteria were grown to an optical density of about 1.00 ($\text{OD}_{600} = 1.00$) for further use.

To conduct the Agar disk-diffusion assays, actively growing cultures of each bacterial strains on Mannitol salt agar (MSA) were inoculated on TSB agar plates. 10 μL bacterial growth culture (cell concentrations were $1.0 \times 10^6 \text{ CFU mL}^{-1}$) was diluted to 1 mL in TSB, and 100 μL of that solution was spread on TSB agar plates to form bacterial lawn covering the plate surface. Then 6 mm (diameter) filter discs were added to the plate surface. Different amounts of PCL-PPB-PCo copolymers and PCL-PPB-PCo-Peni bioconjugates were dissolved in 30 μL water and added to disks. 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 (MICs) of PCL-PPB-PCo-Peni were determined by the following method. Different concentrations of 50 μL aqueous solution of PCL-PPB-PCo-Peni were added to 96-well plates. Then, 150 μL bacterial TSB solution ($\text{OD}_{600} = 1.00$) was added to each well. The bacterial solution without polymers was used as the control. The assay plate was incubated at 37 °C for 12 h. Bacterial growth was detected at OD_{600} and was compared to controls.

LIVE/DEAD bacterial viability assays

Four bacterial strains were inoculated and prepared by a similar procedure described in the above antimicrobial tests. Bacterial solution (1 mL) in TSB media was introduced to PCL-PPB-PCo-Peni bioconjugate (containing 10 μg penicillin-G), respectively. An untreated cell suspension was used as the control. Following 18 h incubation at 37 °C, 1 μL LIVE/

DEAD BacLight (Bacterial Viability Kit; Invitrogen Inc.) was added to the incubation solution. Cells were then imaged using a Leica TCS SP5 CLSM with 63× oil immersion lens. Once excited at 488 nm with argon and helium/neon lasers, bacteria with intact membranes display green fluorescence (emission = 500 nm), while disrupted membranes show red fluorescence (emission = 635 nm).

Bacterial morphology assays

The morphologies of bacteria after incubation with PCL-PPB-PCo-Peni bioconjugates were examined by FE-SEM. In general, 10 μ L of bacterial cell solution was grown on a glass slide in a 12-well plate containing 1 mL of TSB medium at 37 °C overnight. Cell suspensions were diluted to OD₆₀₀ = 1.00. PCL-PPB-Peni bioconjugates (containing 10 μ g penicillin-G) were added to the 1 mL cell stock solution and incubated at 37 °C overnight. A cell suspension without any antimicrobial agents 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 post-fixed with 1% osmium tetroxide at 4 °C for 1 h. The samples were dried, then coated with gold using Denton Desk II Sputter Coater for 120 s and analyzed by FE-SEM. An untreated cell suspension was used as the control.

Toxicity evaluation

Cytotoxicity of PCL-PPB-PCo-7 was further performed using human primary umbilical vein endothelial cells (HUVEC) cultured with vascular cell basal medium containing endothelial cells growth factors (ATCC, Manassas, VA). PCL-PPB-PCo-7 copolymers were added to the cell culture medium when cells were at 70–80% confluency. Aliquots of cell culture medium were taken after 1, 8 and 24 h. LDH levels in the media were measured by LDH-Glo Cytotoxicity Assay kit (Promega, Madison, WI) according to the provided instruction. Medium without copolymers was used as a negative control to determine culture medium background. For maximum LDH release control, Triton X-100 with the final concentration of 0.2% was added to the cell culture medium. The % cytotoxicity was calculated as (experimental LDH level – background) divided by (maximum LDH – background).

Degradation study

The acidic degradation and hydrolytic degradation were carried out in different solutions: diluted HCl/H₂O solution (~0.15 M HCl), PBS solution (pH = 5.5) and PBS solution (pH = 7.4). For example, for acidic degradation, PCL-PPB-PCo-7 copolymers (20 mg) were added into HCl/H₂O (5 mL) in a dialysis bag (Fisher Science, seamless cellulose dialysis tubing, cutoff molecular weight 1000), which was placed into 100 mL HCl/H₂O solution. Next, 1 mL dialysate was taken out at different time to check the UV absorption of cobaltocenium at 270 nm. For keeping a constant volume, 1 mL of fresh diluted HCl/H₂O solution (~0.15 M HCl) was added back to the reservoir after each sampling.

The enzymatic degradation study of PCL-PPB-PCo copolymers was performed using *Pseudomonas* lipase (1 U mL⁻¹) as the enzyme in PBS solution (pH = 7.4) at 37 °C. PCL-PPB-PCo-7 copolymer (20 mg) was added into the PBS solution (5 mL) containing *Pseudomonas* lipase (1 U mL⁻¹) in a dialysis bag (Fisher Science, seamless cellulose dialysis tubing, cutoff molecular weight 1000), which was placed into 100 mL PBS solution

at 37 °C. Next, 1 mL dialysate was taken out at different time to check the UV absorption of cobaltocenium at 270 nm. To keep a constant volume, 1 mL of fresh PBS solution (pH = 7.4) was added back to the reservoir after each sampling.

***In vivo* degradation performance**

All animal procedures were performed in accordance with protocols that are in compliance with the National Institutes of Health Animal Research Advisory Committee Guidelines (NIH OACU) for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina. Six- to eight-week-old female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at the Association for Assessment and Accreditation of Laboratory Animal Care-accredited (AAALAC-accredited) Animal Resource Facility at the University of South Carolina School of Medicine. The mice were placed in 12 h dark/light cycles in temperature-controlled rooms (22–24 °C) as well as given water *ad libitum* and normal chow diet. The mice were used when they were about 12 weeks old. A single dose of PCL-PPB-PCo copolymers (10 mg per kg of body weight) was administered (intraperitoneal injection) into groups of mice. Mice treated with PBS were used as control. Major organs (liver, kidney, lung, and spleen), serum and urine were collected from mice after injection of copolymers from the first and seventh day. The cobaltocenium concentrations in these organs were quantitatively analyzed by ICP-MS.

Hemolysis evaluation

Blood was collected in heparinized tubes after sacrificing the mice and it was diluted by mixing 800 µL of blood with 1000 µL of PBS. The PCL-PPB-PCo-7 copolymers were prepared in PBS buffer at different concentrations. Copolymers (3 mL) were added into 60 µL of the diluted blood samples. The samples were incubated for 1 h at 37 °C followed by centrifugation for 10 minutes at 1500 rpm. Supernatants were collected, and OD was measured at 545 nm to calculate hemolysis rate by using the following equation: $HR = (AS - AN)/(AP - AN)$; where AS, AN, and AP are OD values of the supernatants from test samples, negative control (PBS) and positive control (0.1% Triton-X100), respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
 (a) ^1H NMR and (b) FTIR spectra of PCL-Cl, PCL- N_3 , PCL-PPB- N_3 and PCL-PPB-PCo copolymers.

Fig. 2.

(a) Synthesis of phenylboronic acid-containing polycaprolactone metallopolymers–antibiotic bioconjugates (PCL-PPB-PCo-Peni) via ionexchange. (b) Illustration of synergistic antimicrobial mechanisms of PCL-PPB-PCo-Peni bioconjugates against Gram-negative bacteria. (c) Agar disk-diffusion assays of penicillin-G, PCL-PCo-Peni conjugates and PCL-PPB-PCo-Peni against *E. coli*, *P. aeruginosa*, *P. vulgaris* and *K. pneumoniae*. Aqueous solution of antimicrobial agents (30 µL) with different weights (containing 15–20 µg penicillin-G) was added to disks, and the culture dishes were incubated in the oven at 28 °C for 18 h. (d) OD₆₀₀ values of four bacteria incubated with penicillin-G, PCL-PCo-Peni and PCL-PPB-PCo-Peni conjugates (containing 10 µg mL⁻¹ penicillin-G), respectively. The tryptic soy broth (TSB) solutions of four different bacteria without any antimicrobial agents were used as the control groups.

Fig. 3.

(a) Confocal laser scanning microscopy (CLSM) images and (b) scanning electron microscopy (SEM) images of control group (without copolymer) and PCL-PPB-PCo-Peni bioconjugates against four strains of bacteria. CLSM images using BacLight live/dead stain, green color indicates live cells, and red color indicates dead cells. Bacteria concentration were 1.0×10^6 CFU mL⁻¹.

Fig. 4. Degradability of copolymer PCL-PPB-PCo-7 under three different conditions: (a) acid-catalyzed degradation (0.15 M HCl) and (b) hydrolytic degradation (pH = 7.4 and 5.5), and (c) enzymatic degradation (1 U mL⁻¹ *Pseudomonas* lipase, pH = 7.4).

Fig. 5.
The distribution of cobaltocenium in major organs, serum, and urine after intraperitoneal injection of PCL-PPB-PCo copolymers (PCL-PPB-PCo-7, 10 mg per kg body weight) into groups of mice. Mice treated with PBS were used as control. Five mice were treated in parallel in each group.

Scheme 1.

Synthesis of phenylboronic acid and cobaltocenium-containing polycaprolactone copolymers (PCL-PPB-PCo) *via* ring-opening polymerization and click chemistry.

Table 1

Molecular weight and molar compositions of PCL-PPB-PCo copolymers

Entry	PPB molar percentage	PCo molar percentage	M_n^a (g mol ⁻¹)
PCL-PPB-PCo-1	20%	80%	4500
PCL-PPB-PCo-2	20%	80%	9000
PCL-PPB-PCo-3	20%	80%	15 000
PCL-PPB-PCo-4	20%	80%	25 000
PCL-PPB-PCo-5	20%	80%	35 000
PCL-PPB	100%	0%	15 000
PCL-PPB-PCo-6	10%	90%	15 000
PCL-PPB-PCo-7	15%	85%	15 000
PCL-PPB-PCo-8	20%	80%	15 000
PCL-PPB-PCo-9	30%	70%	15 000
PCL-PCo	0%	100%	15 000

^a M_n calculated *via* ¹H NMR spectra of PCL-Cl polymer.

Table 2

The minimum inhibitory concentration (MIC) of penicillin-G, PCL-PCo-Peni conjugates, and PCL-PPB-PCo-Peni conjugates against Gram-negative bacteria

Compounds	Minimum inhibitory concentration based on penicillin (MIC, $\mu\text{g mL}^{-1}$)			
	<i>E. coli</i> ATCC-22775	<i>P. aeruginosa</i> ATCC-10145	<i>P. vulgaris</i> ATCC-33420	<i>K. pneumoniae</i> ATCC-35596
Penicillin-G	14.8	18.7	15.4	19.8
PCL-PCo-Peni	7.6	8.2	7.5	9.2
PCL-PPB-PCo-Peni	4.1	5.4	4.6	6.5