Antifouling and antimicrobial cobaltocenium-containing metallopolymer double-network hydrogels

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Key Words:
antimicrobial; cobaltocenium; double network hydrogel; metallopolymer

From the Contents

ABSTRACT

Compared with single-network hydrogels, double-network hydrogels offer higher mechanical strength and toughness. Integrating useful functions into double-network hydrogels can expand the portfolios of the hydrogels. We report the preparation of double-network metallopolymer hydrogels with remarkable hydration, antifouling, and antimicrobial properties. These cationic hydrogels are composed of a first network of cationic cobaltocenium polyelectrolytes and a second network of polyacrylamide, all prepared via radical polymerization. Antibiotics were further installed into the hydrogels via ion-complexation with metal cations. These hydrogels exhibited significantly enhanced hydration, compared with polyacrylamide-based hydrogels, while featuring robust mechanical strength. Cationic metallopolymer hydrogels exhibited strong antifouling against oppositely charged proteins. These antibiotic-loaded hydrogels demonstrated a synergistic effect on the inhibition of bacterial growth and antifouling of bacteria, as a result of the unique ion complexation of cobaltocenium cations.

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http://doi.org/10.12336/biomatertransl.2022.02.008

How to cite this article:
Li, H.; Yang, P.; Hwang, J.; Pageni, P.; Decho, A. W.; Tang, C. Antifouling and antimicrobial cobaltocenium-containing metallopolymer double-network hydrogels. *Biomater Transl.* **2022**, *3*(2), 162-171.

Introduction

Hydrogels are a class of ubiquitous materials that have found utilities in a variety of fields.[1-5] Applications of traditional synthetic hydrogels are often limited by their brittleness and fragility. Many new approaches have been developed towards constructing stronger hydrogels, e.g., nanocomposite hydrogels, hydrogels with incorporation of multivalent ions, and slide ring hydrogels.[6-9] Among various hydrogels, double-network (DN) hydrogels, first pioneered by the Gong group[10, 11] and then further demonstrated by other groups,[12-14] stand out, because they can offer high mechanical strength and toughness. DN hydrogels typically comprise two types of polymer network components: (1) cross-linked polyelectrolytes (first network) and (2) loosely cross-linked neutral polymers (second network). The high strength of the DN gels is due to two contrasting network components, wherein the first network serves as sacrificial bonds, while the second network sustains stress by redistributing dissipative energy.

In addition to enhancing mechanical properties, integrating useful functions into the hydrogels is highly desirable to expand the portfolios of DN hydrogels. Metal-containing polymers combine the processibility of an organic polymeric framework with functionalities from metal centers, which in turn promote new properties and activities.[15-21] Regarding the incorporation of metal into a polymer, cationic cobaltocenium moieties have served as new building blocks for functional polymers.[22-25] Cobaltocenium-based polymers have attracted great attention due to their potential applications in catalysis, sensors, energy storage, magnetic materials, healthcare and mechanochemistry.[23, 26-29] Therefore, designing functional metal-containing polymeric hydrogels with enhanced mechanical properties offers scientific significance and practical relevance.

Taking advantage of antimicrobial properties and ion-dependent solubility of cobaltocenium-containing polymers,[24, 30, 31] herein we present the preparation of metallopolymer-based DN hydrogels that exhibit some remarkable

properties beneficial for various applications.[32, 33] Specifically, the DN hydrogels consist of a first network of cationic cobaltocenium with an acrylamide (AM) or 2-hydroxyethyl methacrylate (HEMA) monomer and a second network of AM, which could be fabricated via facile two-step free radical polymerization. These metallopolymer hydrogels significantly enhanced mechanical properties and hydration abilities compared to AM-based counterparts. Their conjugations with β-lactam antibiotics further allowed these metallopolymer hydrogels with desirable biological activities. The antibiotic-containing cationic metallopolymer DN hydrogels exhibited strong antifouling to negatively charged proteins. In addition, these hydrogels also demonstrated robust antibacterial activities due to the synergistic effects of antibiotics and metal-containing building blocks.

Methods

Synthesis of 2-cobaltocenium amidoethyl methacrylate chloride

According to a method we developed earlier,[32] 2-cobaltocenium amidoethyl methacrylate chloride (CoAEMACl) was synthesized by ion-exchange of 2-cobaltocenium amidoethyl methacrylate hexafluorophosphate with IRA-400(Cl) ion exchange resin. The methanol solution of 2-cobaltocenium amidoethyl methacrylate hexafluorophosphate (250 mg/mL, 4 mL) was added into the dispersion methanol solution of IRA-400(Cl) (0.5 g/mL, 20 mL), and stirred for 8 hours. The methanol solution was collected after filtration. The solvent was evaporated under pressure. After freeze-drying, the monomer CoAEMACl was obtained and confirmed by proton nuclear magnetic resonance (^{1}H NMR) (**Additional Figure 1**).

Preparation of hydrogels

Briefly, the first network of chloride-paired cobaltocenium-containing hydrogel (PCoCl hydrogel) was synthesized from an aqueous solution of 0.6 M CoAEMACl and 0.4 M AM (VWR, Atlanta, GA, USA) or HEMA (VWR) containing 4 mol% crosslinking agent, poly(ethylene glycol) dimethacrylate (molecular weight = 3400 g/mol; VWR), and 0.1 mol% initiator ammonium persulfate (99%, Sigma-Aldrich, Florence, SC, USA) in a test tube. Under nitrogen gas, polymerization was kept at 72°C for 12 hours. At 0°C, this hydrogel (first network) was then immersed into another aqueous solution made up of 2 M AM, containing 0.1 mol% N,N′-methylenebisacrylamide, and 0.1 mol% ammonium persulfate for 48 hours under nitrogen. After equilibrium, the second network was subsequently prepared in the presence of the first network. After polymerization, the obtained double-network (PCoCl-DN) hydrogel was immersed in plenty of deionized (DI) water (periodically changed with fresh DI water) for a week to remove unreacted monomers and residual initiators.

Penicillin-conjugated cobaltocenium metallopolymer double-network (PCoPeni-DN) hydrogels were further prepared by ion exchange according to the following: PCoCl-DN hydrogel (dry weight = 50 mg) was first immersed into an aqueous solution of penicillin-G sodium salt (2 mg/mL; VWR) for 48 hours at 25°C. Then, the resultant hydrogel was kept in a large amount of DI water for at least 2 days. The water was changed every 4 hours to remove unbounded penicillin-G and free sodium chloride. The penicillin-G mass in PCoPeni-DN hydrogel 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 an ultraviolet-visible spectrophotometer (DS5 Dual Beam, Fulton, MD, USA) at 250 nm and a standard penicillin calibration curve.

General characterization

^{1}H NMR (300 MHz) spectra were recorded on a Varian Mercury 300 spectrometer (Palo Alto, CA, USA) with tetramethylsilane as an internal reference. The tensile tester (5543A, Instron, Boston, MA, USA) was used to carry out uniaxial stretching on specimens of 50 mm length, 5 mm breadth, and 3 mm height at a strain rate of 10%/minutes. Compression tests were carried out with an Instron 5543A. Cylindrical gel samples with a height of about 5 mm and a diameter of about 10 mm were used for compression tests. The compression rate was 0.1%/min.

The equilibrium water content (*EWC*, wt%) of hydrogels was assessed by comparing weight of the wet sample (m_w) and dry sample (m_d).[34] The *EWC* of the samples was calculated by using the following equation:

$$EWC(\%) = \frac{m_w - m_d}{m_w} \times 100\% \quad (1)$$

According to a reported method,[35, 36] the hydration ability of polymer networks was evaluated by differential scanning calorimetry (DSC; Q2000, TA Instruments, New Castle, DE, USA). The weight ratio ($W_{\text{non-freezable}}$) of non-freezable water to polymer in the hydrogel was calculated by using the equation (2). The larger value of non-freezable water often shows the higher hydration binding affinity of polymer chains.

$$W_{\text{non-freezable}} = \frac{EWC - \frac{\Delta H_f}{\Delta H_w} \times 100}{w_{\text{polymer}}} \quad (2)$$

Where w_{polymer} is the weight percentage of polymer in hydrogels, ΔH_f is the enthalpy associated with the melting of freezable water and free water in a hydrogel as measured by DSC, and ΔH_w is the enthalpy for the melting of bulk water by DSC. The determination of ΔH_f was done by integrating all endothermic peaks in the range of approximately –5°C to 10°C.

The weight of anionic penicillin-G (mg) per 1.0 g penicillin-

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conjugated cobaltocenium metallopolymer double-network (PCoPeni-DN) hydrogel (C_{Peni}) can be calculated by using the following equation:

$$C_{Peni} = \frac{(m_2 - m_1) \times \frac{M_{pNa} - M_{Na}}{M_{pNa} - M_{NaCl}}}{\frac{m_2}{100 - EWC}} \times 10\ (mg/g) \quad (3)$$

Here, m_1 (g) and m_2 (g) are the weight of dried PCoCl-DN and PCoPeni-DN hydrogels respectively. M_{pNa}, M_{NaCl} and M_{Na} are the molecular weight of penicillin sodium, sodium chloride, and sodium, respectively. *EWC* is the equilibrium water content of the PCoPeni-DN hydrogel.

Protein fouling test of hydrogels

Hydrogels were immersed in fluorescein isothiocyanate-conjugated bovine serum albumin (BSA-FITC) solution (0.5 mg/mL in phosphate buffered saline (PBS) solution; Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 2 hours to allow protein adsorption. Then, the hydrogels were rinsed gently with PBS solution and DI water to remove any loosely bound BSA-FITC on the surface. The hydrogel surface was imaged under an inverted fluorescence microscope (Nikon eclipse LV, Tokyo, Japan), and the fluorescent intensity was subsequently quantified and analyzed using an ImageJ software (Version 1.8.0.172, National Institutes of Health, Bethesda, MD, USA).[37]

Bacterial adhesion and viability on the hydrogel surface

Escherichia coli (*E. coli*, ATCC 25922) was purchased from American Type Culture Collection (Manassas, VA, USA). A spread plate method[38] was used to quantify bacterial adhesion and viability on the surface of hydrogels. 15 mL of tryptic soy broth and 50 μL of log-phase cultures of *E. coli* were mixed in a sterile conical tube, and then the bacterial suspension was obtained. The hydrogels were punched into 3 mm disks and washed with sterile PBS three times. These hydrogel disks and control samples were placed into individual wells of a sterile 12-well plate in triplicates, followed by the addition of 0.5 mL of the bacterial suspension to each well and incubated at 37°C with continuous shaking at 150 r/min. After 24 hours, these hydrogels were gently washed thrice with sterile PBS to remove loosely bound bacteria. Then the *E. coli* cells adhered on the hydrogel surface were washed with 1 mL of sterile PBS solution under mild ultrasonication for 8 minutes. The formed bacterial suspension was placed in a serial tube, followed by 1000-fold serial dilution. 100 μL aliquots of the serially diluted suspension were spread onto the triplicate solid agar. After incubation of the plates at 37°C for 24 hours, the number of viable cells (colonies) were counted manually, and the results were expressed as the relative viability, defined as the percentage of viable cells on the hydrogel relative to that on the substrate. There are two control samples: one is a glass slide; the other includes a glass slide loaded with penicillin-G sodium salts whose weight is equal to the weight of penicillin sodium salts in hydrogel disks.

Bacterial adhesion and growth inhibition assay

Based on reported methods,[39, 40] propidium iodide and SYTO 9 (live/dead BacLight viability kit, Life Technology, Carlsbad, CA, USA) were used to label bacterial cells on as-prepared hydrogels. The bacterial suspension was obtained by mixing 15 mL of tryptic soy broth and 50 μL of log-phase cultures of *E. coli*. Three hydrogel disks of 3 mm were placed into individual wells of a sterile 12-well plate, followed by the addition of 0.5 mL bacterial suspension to each well for incubation at 37°C with continuous shaking at 150 r/min. After 1 day, both the concentration of live *E. coli* in solution and the accumulated bacteria on the surface of hydrogels were measured. To determine the concentration of live bacteria in solution, the bacterial culture was carefully removed from each well for optical density at 600 nm reading with a spectrophotometer (MR9600, Accuris, Edison, NJ, USA). To analyze the density of bacteria accumulated on hydrogel surfaces. These hydrogel disks were gently washed with DI water to remove any loosely bound bacteria, then the bacteria adhered on each hydrogel disk surface was obtained by using 1 mL of sterile PBS solution to clean the sample under mild ultrasonication for 8 minutes. The obtained bacterial suspension was stained with a live/dead BacLight bacterial viability kit and observed using confocal laser scanning microscopy (Leica TSC SP5, Wetzlar, Hesse-Darmstadt, Germany). Moreover, the concentration of live bacteria adhered on each hydrogel disk surface was obtained from the optical density reading.

Statistical analysis

All experiments were performed at least in triplicate. Data are presented as mean ± standard deviation (SD) by Origin software (Origin Pro 8; OriginLab Corp., Northampton, MA, USA).

Results and Discussion

Preparation of hydrogels by radical polymerization

Cobaltocenium is an oxidized, cationic form of neutral cobaltocene.[41] It is highly stable toward harsh conditions such as oxidation and acidic/basic environments.[23, 27] In addition, cobaltocenium-containing polymers exhibit certain unique properties such as ion-pairing ability and antimicrobial activities.[24, 32] Thus, we chose this metal cation as a key functional moiety for integration into hydrogels. **Figure 1** illustrates the preparation of cobaltocenium-containing DN hydrogels via free radical polymerization and subsequent ion exchange. We first demonstrated that CoAEMACl and poly(ethylene glycol) dimethacrylate can be used as a monomer and a crosslinker respectively to prepare a single-network hydrogel (SN-1). Due to the presence of cobaltocenium groups and PEG segments, SN-1 has a hydrophilic polymer network that can reach an EWC as high as 97.5% (**Table 1**). However, SN-1 is brittle with low compressive stress.

To construct metallopolymer DN hydrogels,[33, 42] SN-1 was designed as the 1st polymer network while polyacrylamide (PAM) was subsequently prepared as the 2nd network in the presence of the first network. As shown in **Table 1**, the compressive stress of the resultant hydrogel (DN-1) (~0.47 MPa) was higher than that of SN-1, but less than that of an SN hydrogel (SN-2) based on PAM alone (**Additional Figure 2**). This result showed that high strength hydrogels

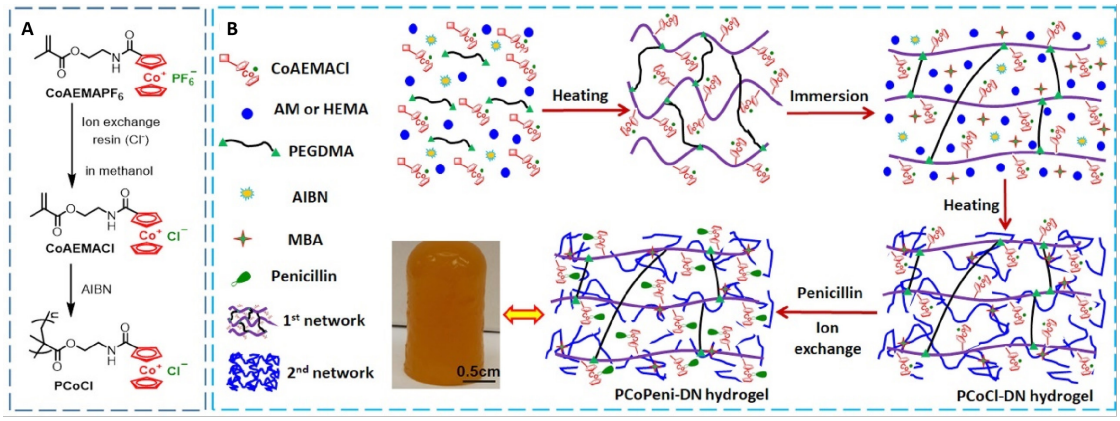


Figure 1. (A) Synthesis and homo-polymerization of 2-cobaltocenium amidoethyl methacrylate chloride (CoAEMACl). (B) Preparation of penicillin-conjugated cobaltocenium metallopolymer double-network (PCoPeni-DN) hydrogel via free radical polymerization and ion exchange. AIBN: azobisisobutyronitrile; AM: acrylamide; CoAEMAPF$_6$: 2-cobaltocenium amidoethyl methacrylate hexafluorophosphate; HEMA: hydroxyethyl methacrylate; MBA: N,N′-methylenebisacrylamide; PCoCl: chloride-paired cobaltocenium polymer; PEGDMA: poly(ethylene glycol) dimethacrylate.

Table 1. The network compositions of hydrogels, and their EWC and mechanical properties

Sample	First network	Second network	Penicillin-G content (mg/g)	EWC (wt %)	σ_{max} (MPa)	λ_{max} (%)
SN-1	PCoCl	–	–	97.5	0.014	37
SN-2	–	PAM	–	94.3	0.55	91
DN-0	PHEMA	PAM	–	72.4	0.64	53
DN-1	PCoCl	PAM	–	95.1	0.47	49
DN-2	PAM	PAM	–	93.2	3.74	85
DN-3	P(CoCl-AM)	PAM	–	94.3	3.22	74
DN-4	P(CoCl-HEMA)	PAM	–	87.9	1.86	66
DN-5	P(CoCl-AM)	PAM	7.41	91.7	5.81	79
DN-6	P(CoCl-HEMA)	PAM	6.85	83.5	2.45	68

Note: C_{Peni}: penicillin content; DN: double-network; EWC: equilibrium water content; P(CoCl-AM): poly(chloride-paired cobaltocenium-acrylamide); P(CoCl-HEMA): poly(chloride-paired cobaltocenium-hydroxyethyl methacrylate); PAM: polyacrylamide; PCoCl: chloride-paired cobaltocenium metallopolymer; PHEMA: poly(2-hydroxyethyl methacrylate); SN: single-network; λ_{max}: maximum strain rate; σ_{max}: maximum compressive stress.

were not achieved by using this particular DN structure, a property which might be related to the stiff macromolecular chains and the low molecular weight of the 1st polymer network due to the nature of PCoCl homopolymers.[24] In order to get higher compressive stress, copolymerization of CoAEMACl and AM was carried out to serve as the 1st polymer network. The DN-3 hydrogel achieved a stress of 3.22 MPa and a strain of 74%, indicating excellent mechanical properties. As controls, DN-0 was prepared with the first network of poly(2-hydroxyethyl methacrylate) (PHEMA) and the second network of PAM, while DN-2 with both networks being based only on PAM. The stress of DN-3 was slightly less than that of DN-2, but far higher than SN-1, SN-2, DN-0 and DN-1. The EWC of DN-3 was approximately 94.3%, which is very close to that of DN-1 (95.1%) and DN-2 (93.2%). Our results also showed that with higher contents of cobaltocenium moieties, there was a higher corresponding EWC of hydrogels, further implying that the cobaltocenium chloride groups have a strong hydration capacity.[43]

Considering HEMA polymers as a good antifouling agent,[44, 45] a hydrogel DN-4 was also prepared using HEMA and PCoCl as the co-monomers in the 1st network. The hydrogel DN-4 has a stress of 1.86 MPa and a strain of 66%, and the EMC of DN-4 was approximately 90.3%. It was found that the use of HEMA in the 1st polymer network decreased the EMC in comparison to DN-3, a result mainly attributed to the more hydrophobic nature of PHEMA backbone.

Taking advantage of the ion-pairing capability of cobaltocenium, one of the most common β-lactam antibiotics, penicillin-G, was loaded into metallopolymer-containing DN hydrogels (DN-3 and DN-4). The resultant PCoPeni-DN

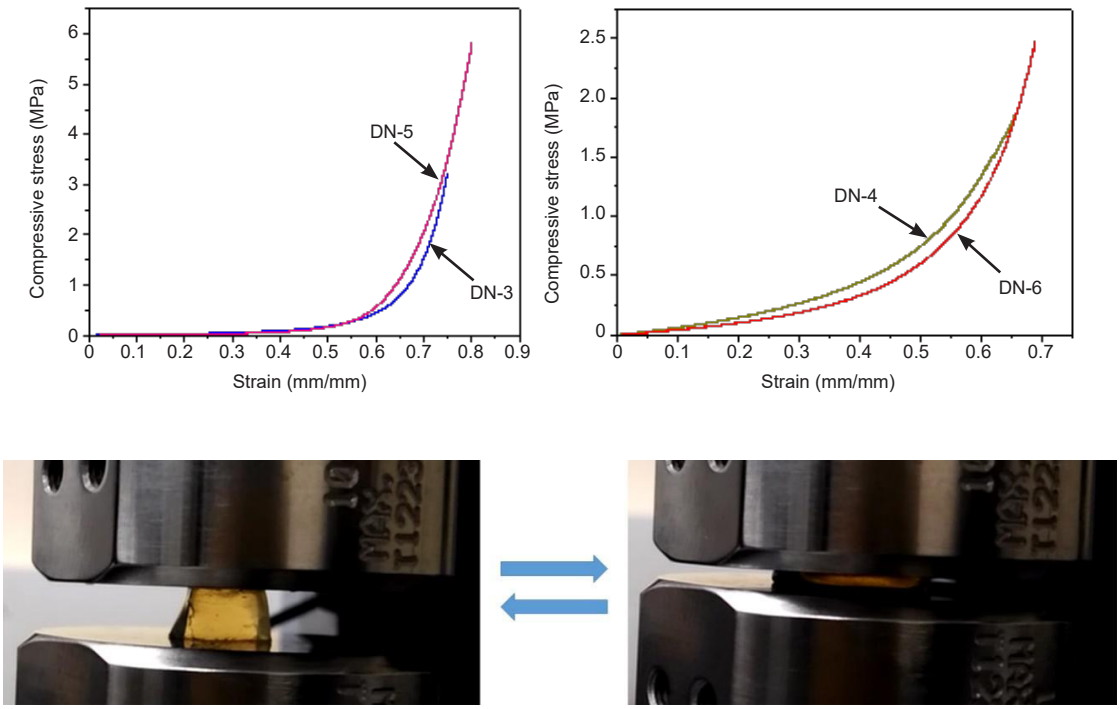


Figure 2. Compression stress-strain plots of PCoCl-DN hydrogels (DN-3 and DN-4) and PCoPeni-DN hydrogels (DN-5 and DN-6) (upper). Photographs showing reversible compression of hydrogel (DN-5) (lower). A video of compression testing is included in the Additional Video 1. DN: double network; PCoCl-DN: chloride-paired cobaltocenium-containing double-network; PCoPeni-DN: penicillin-conjugated cobaltocenium metallopolymer double-network.

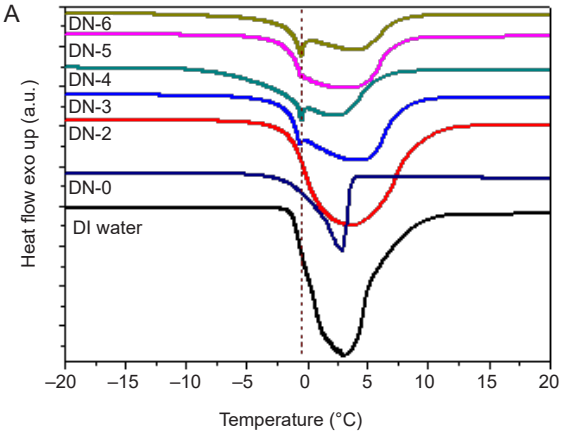
hydrogels (DN-5 and DN-6) were immersed in DI water to remove excess free penicillin. Ultraviolet-visible absorption was used to detect the content of penicillin in hydrogels. In general, approximately 48 hours was enough to achieve equilibrium for the removal of excess penicillin (**Additional Figure 3**). The penicillin-G contents in DN-5 and DN-6 hydrogels were 7.41 mg/g and 6.85 mg/g respectively. As seen in **Figure 2**, the compression stress and strain of DN-5 was 5.81 MPa and 79%, while DN-6 has 2.45 MPa and 68% respectively. Compared with DN-3 and DN-4 hydrogels, the hydrogels with penicillin have even higher mechanical strength, partially due to improved hydrogen bonding interactions with the introduction of penicillin. Moreover, the tensile test of PCoCl-DN and PCoPeni-DN hydrogels was also carried out (**Additional Figure 4**). All DN hydrogels exhibited an elastic tensile profile (i.e., no thermoplastic yielding observed) and the tensile strength was less than 32 kPa.

Hydration of cobaltocenium-containing double-network hydrogels

Figure 3 shows DSC curves of different DN hydrogels. The DSC curve of DI water was also obtained for comparison. The DN-0 hydrogel containing PHEMA and PAM showed only one endothermic peak. The PAM-based DN-2 hydrogel (without cobaltocenium) showed a single endothermic peak, and the thermal transition of DN-2 was similar to that of the ice/water transition for bulk DI water over a broad range of temperatures. Interestingly, each of other four hydrogels (DN-3, DN-4, DN-5 and DN-6) containing cobaltocenium has two endothermic peaks: the broad peak at higher temperature is caused by free water (bulk-like water); the other peak below 0°C is likely attributed to freezable bound water.[35] These results showed that the introduction of cobaltocenium groups depressed the melting point of water below 0°C, most likely due to the improvement of hydration for hydrogels.

It is well known that the non-freezable water is closely related to the hydration binding affinity of polymer chains.[35, 36] DSC was used to obtain the non-freezable water in the hydrogels in order to further understand the hydration abilities of hydrogels. According to ΔH_f and *EWC*, the weight ratio ($W_{\text{non-freezable}}$) of non-freezable water to polymer in hydrogels was obtained, as shown in **Figure 3**. Not surprisingly, the DN hydrogel containing a PHEMA network (DN-0) has the highest $W_{\text{non-freezable}}$ due to the excellent hydration of PHEMA. The $W_{\text{non-freezable}}$ of DN-3, DN-4, DN-5 and DN-6 was 1.26, 1.21, 1.19 and 1.16, respectively, each of which was significantly larger than that of DN-2 (0.72), indicating that the introduction of cobaltocenium groups appreciably improved the hydration of hydrogels.

A



B

Sample	ΔH_f (J/g)	$W_{non\text{-}freezable}$
DN-0	115.9	1.39
DN-2	302.5	0.72
DN-3	297.9	1.26
DN-4	251.2	1.21
DN-5	279.7	1.19
DN-6	220.3	1.16

Figure 3. (A) DSC curves of different DN hydrogels and DI water. (B) Enthalpy change (ΔH_f) associated with the melting of freezable water per weight of a hydrogel measured by DSC. The enthalpy change for the melting of bulk water is ΔH_w = 335.2 J/g. ΔH_f: the enthalpy associated with the melting of freezable water and free water in a hydrogel; ΔH_w: the enthalpy for the melting.

Protein adsorption on cobaltocenium-containing double-network hydrogels

The hydration of hydrogels is one of the critical factors dictating their antifouling properties.[46] Antifouling is the ability of natural or synthetic material surfaces to reduce or prevent the adhesion or attachment of biomolecules.[47] One of the greatest challenges for ionic hydrogels is to prevent the adsorption of proteins containing opposite charges to the hydrogels, simply due to the presence of strong ionic interactions. To test the antifouling performance of cobaltocenium DN hydrogels, the adsorption of BSA-FITC onto prepared hydrogels (as described above) was evaluated after immersing the hydrogels into a BSA-FITC solution. Fluorescence microscopy images and average fluorescence intensities of cobaltocenium-containing hydrogels are shown in **Figure 4**. In comparison with DN-2, the fluorescence intensity of adsorbed BSA on DN-3 and DN-5 surfaces increased to some extent. This observation indicated that these hydrogels cannot reduce BSA fouling due to the presence of electrostatic interactions, although the introduction of cobaltocenium groups make the polymer networks of hydrogels more hydrated.[48-51] However, the fluorescence intensity of adsorbed BSA on DN-4 and DN-6 surfaces significantly decreased, by approximately 13% and 19% respectively compared to that on DN-2. This indicated a good resistance to protein adsorption, primarily due to the presence of antifouling HEMA polymers in the networks. The installation of bulky penicillin did not have a significant reduction of net positive charges on hydrogels, which was confirmed by a zeta potential study. Compared to the zeta potential of a cobaltocenium homopolymer PCoCl at +45 mV, the positive potential of a penicillin-loaded cobaltocenium homopolymer PCo-Peni slightly decreased to +35 mV.

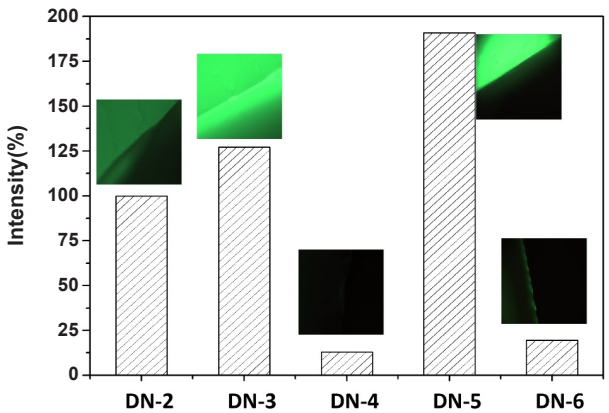


Figure 4. Average fluorescence intensities of cobaltocenium-containing DN hydrogel surfaces after immersing into 0.5 mg/mL of BSA-FITC solution; fluorescence microscopy images are attached. BSA-FITC: fluorescein isothiocyanate-conjugated bovine serum albumin; DN: double-network.

Bacterial adhesion and viability on double-network hydrogels

Antibiotics have been used for decades to treat bacteria-induced infections. Increasing antibiotic resistance by bacteria has prompted research on other therapeutic approaches. Cationic polymers are one of common biomaterials designed for killing bacteria.[52-56] A combination of both cationic polymers and antibiotics could potentially offer synergistic effects, as recently demonstrated in the case of a bioconjugate of cobaltocenium homopolymers and antibiotics.[24] A spread-plate method was utilized for evaluating adhesion and viability of *E. coli* on the surface of cobaltocenium-containing metallopolymer DN hydrogels (**Figure 5**). In comparison with controls (bare glass slides) (**Figure 5A**), all other surfaces exhibited a decrease in bacterial adhesion (**Figure 5B–G**). Different from protein fouling, DN-3 showed bacterial adhesion with a viable fraction of 30%, significantly lower than DN-2 (at 52%) (**Figure 5H**). The result illustrated that the cationic cobaltocenium polymer in DN-3 could damage or kill bacteria, leading to the reduction of bacterial adhesion. The DN-4 surface exhibited higher antibacterial efficiency than DN-3, likely due to both antifouling and antibacterial activities. As seen in **Figure 5B**, with the addition of penicillin only (5 μg/mL), many bacteria still adhered to the surface of glass slides with a viable fraction of 28%. For DN-5 and DN-6 hydrogels that were conjugated with an equivalent amount of penicillin as used alone, bacteria were eradicated completely without any visible colonies on the plates (**Figures 5F** and **G**), exhibiting remarkable antibacterial efficiency. Compared to antibiotic alone and hydrogels with only cobaltocenium, the enhanced efficacy of hydrogels loaded with both cobaltocenium and antibiotic further indicated that there is a synergistic effect on killing bacteria, which is highly desirable in developing new therapeutic treatments on frequently occurring bacterial outbreak.[24]

Bacterial growth inhibition assay

Two bacterial growth studies with DN hydrogels in culture media were carried out: (1) How are bacteria in the cultural medium affected by DN hydrogels? (2) How are bacteria on the surfaces of DN hydrogels? After incubation for 24 hours, the growth of live *E. coli* in culture media with various hydrogels was determined by measuring optical density at 600 nm (**Figure 6A**). DN-2 without cobaltocenium featured the most rapid bacterial growth, similar to the control group. There was a slight decrease in bacterial growth for DN-3 and DN-4. However, both cobaltocenium and penicillin-containing metallopolymer DN hydrogels (DN-5 and DN-6) exhibited outstanding bacterial inhibition with optical density reductions of 95.9% and 92.3%, respectively, which was again mainly due to the synergistic effect of penicillin-G and cobaltocenium from the hydrogels.[48]

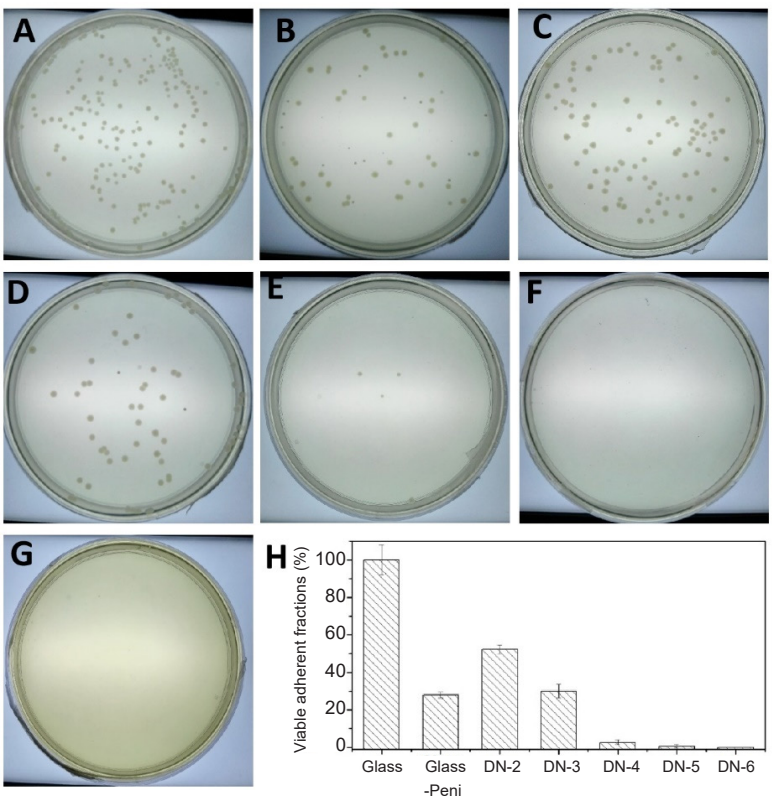


Figure 5. (A–G) Images of *Escherichia coli* (*E. coli*) colonies of viable adherent bacterial cells on the surfaces of glass slides (A), glass slides with penicillin-G sodium salts (B), DN-2 (C), DN-3 (D), DN-4 (E), DN-5 (F) and DN-6 (G). (H) Viable adherent fractions of E. coli on the surfaces of samples from the above spread plates. DN: double-network; Peni: penicillin-G.

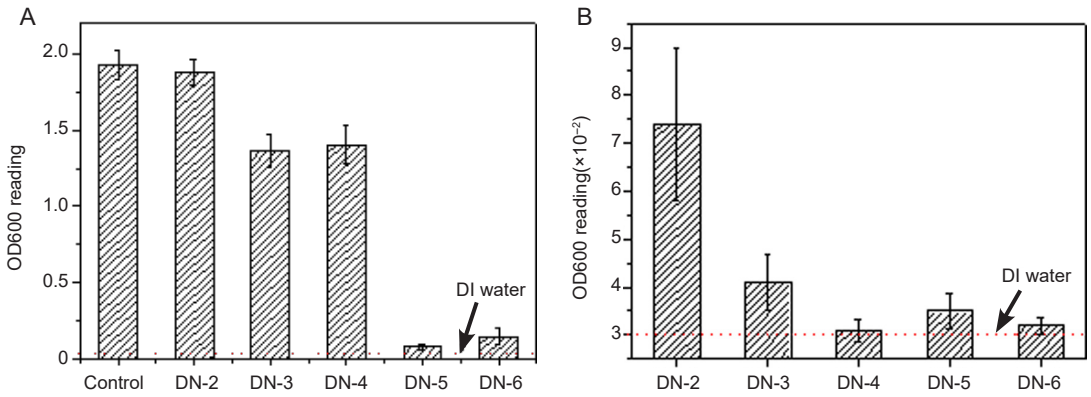


Figure 6. (A) Optical density at 600 nm (OD 600) of Escherichia coli (*E. coli*) culture solution upon 24 hours incubation with different hydrogels. (B) OD600 values of the *E. coli* suspension washed from different hydrogels. OD 600 of DI water is about 0.03. DI: deionized; DN: double-network.

To evaluate the viability of bacteria on the surfaces of hydrogels, attached bacterial cells on hydrogels were further examined with confocal laser scanning microscopy using live/dead stained *E. coli* cells. **Additional Figure 5** shows confocal laser scanning microscopy images of live and dead bacteria cells washed from the hydrogel surfaces after fluorescent staining of the bacteria. For DN-2, most of bacterial cells fluoresced green, indicating that these cells were viable. Compared with DN-2, a majority of dead cells (stained red) appeared on the DN-3 surface. Significantly, most cells were dead with very few live cells appearing on the DN-5 surface after loading with penicillin-G. This observation was consistent with the results of the bacterial adhesion and viability from the spread plate study. For DN-4 and DN-6, few live and dead bacteria cells were observed on their surfaces because of their good antifouling and antibacterial properties. Consistent results were also confirmed by the optical density at 600 nm of the *E. coli* suspension washed from the hydrogels (**Figure 6B**).

Conclusions

In summary, we have successfully prepared strong PCoCl-DN hydrogels, followed by a conjugation of antibiotic penicillin with the hydrogels via ionic complexation to yield dual functions of antifouling and antimicrobial efficacy. The as-prepared PCoCl-DN and PCoPeni-DN hydrogels have excellent mechanical strength and high hydration ability, as evidenced by compression test and DSC measurement. The strong antifouling cobaltocenium-containing hydrogels (DN-4 and DN-6) resisted adsorption of negatively charged bovine serum albumin and adhesion of *E. coli*. Additionally, PCoPeni-DN hydrogels (DN-5 and DN-6) not only exhibit outstanding bacterial inhibition, but also damage or kill bacteria with remarkable antibacterial efficiency. This class of metallopolymer hydrogels may open new pathways for biomedical applications.

Author contributions

CT conceived the design and methodology and supervised the project; HL performed synthesis and characterization of hydrogels as well as antifouling experiments; PY and PP performed antimicrobial tests; JH performed data analysis; HL, PY, JH, AWD, and CT wrote the manuscript. All authors approved the final version of the manuscript.

Financial support

The study was supported by the National Institutes of Health (No. R01AI120987).

Acknowledgement

None.

Conflicts of interest statement

The authors declare no competing financial interests.

Additional files

Additional Figure 1: ^{1}H NMR spectrum of monomer CoAEMACl.

Additional Figure 2: Compression stress-strain plots of hydrogels SN-1, SN-2, DN-1 and DN-2.

Additional Figure 3: Release profile of excess penicillin-G from hydrogels as a function of time in deionized water.

Additional Figure 4: Tensile strength-strain plots of hydrogels DN-3, DN-4, DN-5 and DN-6.

Additional Figure 5: Confocal laser scanning microscopy images of live and dead *Escherichia coli* cells washed from the surfaces of glass (control) and the surface of hydrogels DN-2, DN-3, DN-4, DN-5 and DN-6.

Additional Video 1: A video of compression testing of PCoPeni-DN hydrogels (DN-5).

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Received: April 7, 2022
Revised: May 19, 2022
Accepted: June 13, 2022
Available online: June 28, 2022