Tannic acid-inspired, self-healing, and dual stimuli responsive dynamic hydrogel with potent antibacterial and anti-oxidative properties

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Due to their intrinsic injectable and self-healing characteristics, dynamic hydrogels, based on dynamic covalent bonds, have gained a great attention. In this study, a novel dynamic hydrogel based on the boronic ester dynamic covalent bond is facilely developed using phenylboronic acid-modified hyaluronic acid (HA–PBA) and plant-derived polyphenol-tannic acid (TA). The dynamic hydrogel gelated quickly under mild conditions and had favorable viscoelastic properties with good self-healing and shear-thinning capabilities. Moreover, the simultaneous utilization of TA as a reductant for the green synthesis of silver nanoparticles (AgNP) inspired the preparation of a TA-reduced AgNP hybrid dynamic hydrogel with potent and broad-spectrum antibacterial activities. The dynamic hydrogels could also be applied for pH- and reactive oxygen species (ROS)-responsive release of loaded protein molecules without showing evident cytotoxicity and hemolysis in vitro. In addition, the dynamic hydrogels showed the anti-oxidative properties of high free radical and ROS scavenging capacity, which was verified by the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay and ROS fluorescence staining. Overall, this novel class of cytocompatible, self-healing, dual stimuli responsive, antibacterial, anti-oxidative, and injectable hydrogels could be promising as a wound dressing for chronic wound healing.

1. Introduction

Wound dressings are important clinical management tools for promoting wound healing. Among different kinds of wound dressings, injectable hydrogels have exhibited numerous advantages. They can not only maintain the moisture in the wound and allow oxygen permeability, as other hydrogels do, but also fill in wounds of irregular shapes and be in situ loaded with diverse drugs and responsively release those drugs under environmental stimuli, such as pH and reactive oxygen species (ROS). It is well known that patients with diabetes are likely to suffer from impaired wound healing that is complicated by bacterial wound infection. The bacterial proliferation in wounds shifts the skin microenvironment from a normal acidic pH (4.5–5.5) to a neutral or alkaline pH (7.15 to 8.9), which triggers detrimental effects, including biofilm stimulation and prolonged inflammation. Injectable hydrogels incorporated with a myriad of antibacterial agents have been developed to fight those infections. Although antibiotics could be sustainably released from developed hydrogels in the wound site, resulting in long-lasting effect, they still lack efficacy against infections caused by the antibiotic-resistant bacterial strains, such as methicillin-resistant Staphylococcus aureus (MRSA) that is widely present in chronic wounds. To solve this challenge, injectable hydrogels containing novel antibacterial agents, such as antimicrobial peptides, antimicrobial polymers, and metallic nanoparticles, have been developed and have shown excellent antibacterial activity against antibiotic-resistant bacterial strains in vitro and in vivo. However, despite the anti-infective attributes, the wound healing was still impeded, as most of those hydrogels did not address the associated ROS elevation in chronic wounds, which is caused by a combination of mechanisms that lead to...
increased ROS production and reduced antioxidant defense.\(^{13,14}\) The excessive ROS in wounds have been found to induce strong negative impacts on the functions of immune cells, fibroblast, and endothelial cells, thereby retarding the wound tissue regeneration.\(^{14,15}\) There are only a few studies reported on the development of hydrogels with dual actions that target both wound infection and ROS imbalance concomitantly.\(^{2,16,17}\) Additionally, many biomolecules, such as the angiogenic factors including the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are known to wound healing.\(^ {18}\) Antibacterial and anti-oxidative hydrogels loaded with such biomolecules that can be responsively released under pH or ROS change in the wound could enhance wound healing efficacy but are yet to be developed.\(^ {19}\)

Dynamic hydrogels based on dynamic bonds, such as the imine bond, hydrazone bond, and coordination bond, are injectable and present the self-healing property that originates from the reversibility and equilibrium of those dynamic bonds.\(^ {20}\) Such self-healing hydrogels are particularly useful as wound dressings, as they can maintain the gel integrity and reduce the gel fracture under mechanical force, thereby continuously supporting wound healing.\(^ {21}\) Up to date, a variety of dynamic hydrogels based on imine and coordination bonds have been designed as wound dressings.\(^ {2,17,21,22}\) Among all types of dynamic hydrogels, we are interested in the boronic ester bond-based dynamic hydrogels because of their unique properties. The boronic ester bond is rapidly formed without a catalyst between boronic acid and 1,2-diols or 1,3-diols in solutions under ambient conditions, and boronic ester bond-based dynamic hydrogels are receiving increased attention in the biomedical field due to their good bio-compatibility and inherent multi-stimuli responsiveness.\(^ {4,21–25}\) However, boronic ester bond-based dynamic hydrogels with antibacterial activity have been rarely reported.\(^ {26,27}\)

Silver nanoparticles are promising antimicrobial agents for treating infections due to their broad-spectrum bactericidal properties and effectiveness towards multi-resistant bacterial strains.\(^ {28}\) They are often chemically prepared by the reduction of silver ions using various reductants, such as sodium borohydride or ascorbic acid, followed by the addition of stabilizing agents.\(^ {29}\) However, the biogenic synthesis of silver nanoparticles using plant extracts \emph{via} the so called "green chemistry" procedure is getting more popular recently because of its simple and eco-friendly characteristics and concurrent stabilization effect from the plant extracts.\(^ {30}\) Synthesized AgNP can be further dispersed in three-dimensional hydrogel networks to decrease the initial burst leakage and avoid long-term aggregation, thereby reducing the cutaneous toxicity.\(^ {12}\) Many functional hydrogels containing AgNP have been reported with satisfactory biocompatibility and antibacterial properties.\(^ {28,31}\) A wound dressing comprised of green synthesized AgNP and an injectable hydrogel will be greatly appreciated in view of the combinatorial benefits of such a system.

Tannic acid (TA) is a natural, plant-derived polyphenol and has been widely used in biomaterial design, including surface functionalization, protein modification, and biomaterial cross-linking.\(^ {32}\) TA has good biocompatibility and is affirmed as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA).\(^ {33}\) It also possesses antibacterial, anti-oxidative, and anti-inflammatory properties and has been applied in burn wound treatment.\(^ {16}\) These features have influenced the development of hydrogels containing TA as a key precursor, which simply endows the hydrogels with multifunctional properties that are fit for various biomedical applications.\(^ {16,32,34}\) Besides, the galloyl and catechol groups in TA have redox potential, facilitating the utilization of TA as a green chemistry approach for the preparation of metallic nanoparticles, such as silver and gold nanoparticles.\(^ {35–38}\)

In this work, TA acts with three functions: the reductant, the hydrogel precursor, and the protein binder. An injectable dynamic hydrogel based on the complexation between phenylboronic acid (PBA) grafted HA and TA, with \emph{in situ} hybridization of TA reduced AgNP, is facilely fabricated. The dynamic hydrogel dispersed with AgNP showed pH and ROS dual stimuli responsiveness for protein drug delivery as well as ROS scavenging capacity, which are expected to promote wound healing. The dynamic hydrogel was biocompatible \emph{in vitro} and has shown potent and broad-spectrum antimicrobial activity, enabling its use as a bioactive wound dressing.

### 2. Materials and methods

#### 2.1 Synthesis of hyaluronic acid–phenylboronic acid polymer conjugates (HA–PBA)

The HA–PBA polymer conjugate was prepared by conjugating 2-aminophenyl boronic acid (PBA, Alfar Aesar) to HA (290 kDa, Bloomage Biotech) using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC)/hydroxybenzotriazole (HOBt) as the coupling agent (Fig. 1A).\(^ {39}\) In general, 100 mg of HA (0.25 mmol) was first dissolved in 20 mL of de-ionized (DI) water followed by the addition of 43 mg of PBA (0.25 mmol) under constant stirring. Next, 48 mg of EDC (0.25 mmol, Sigma) and 34 mg of HOBt (0.25 mmol, Oakwood Chemical) that were dissolved together in

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200 \mu L of dimethyl sulfoxide (DMSO, Fisher) were added to the above mixture. Then the pH of the solution was adjusted to 5 using a 1 N HCl (Fisher Chemical) solution. The reaction mixture was stirred at room temperature for 48 h to ensure high conjugation efficiency. After that, the mixture was transferred to a dialysis bag with a 6–8 kDa molecular weight cut-off (MWCO) (Spectrum) and dialyzed against DI water for 3 days at room temperature, with the water changed every 12 hours. The dialyzed solution was freeze-dried in a lyophilizer (model FreeZone, Labconco) for 2 days to acquire the HA–PBA polymer conjugate. The conjugates were stored at \(-20^\circ\text{C}\) before use.

2.2 Proton nuclear magnetic resonance (\(^1\)H NMR) analysis of HA–PBA conjugates

\(^1\)H NMR analysis was conducted on a 500 MHz Bruker NMR system and analyzed with Topspin 4.0 software. The polymer conjugate was dissolved in D\(_2\)O (Acros Organic) at 4 mg mL\(^{-1}\) for NMR acquisition, with the chemical shifts referring to the solvent peak of D\(_2\)O at 4.78 ppm at 25 \(^\circ\text{C}\). The substitution degree was determined by using the ratio of the integral of aromatic protons from the conjugated phenylboronic acid group (between 7.5–8 ppm, 4H, \(-C_6H_4\)) to the integral of the HA methyl proton peak (at 2.0 ppm, 3H, \(-CH_3\)).

2.3 Fabrication of injectable HA–PBA–TA dynamic hydrogels with or without encapsulated silver nanoparticles

The HA–PBA–TA dynamic hydrogels were prepared by mixing 2 wt% HA–PBA solution with 1 wt% TA (Chem-Impex) solution in a 3:1 volume ratio at room temperature. The gelation was caused by the boronic ester dynamic bond formation between the PBA groups in HA–PBA and the gallate/catechol groups in TA. The prepared hydrogel was referred to as the blank gel. To prepare the dynamic hydrogel with encapsulated AgNP, the pH of a 1 wt% TA solution was adjusted to neutral (pH = 7) with a 0.5 M \(\text{Na}_2\text{CO}_3\) (Fisher Chemical) solution, and the resultant solution was mixed with an equal volume of 1 wt% silver nitrate (Sigma) solution to prepare the TA reduced AgNP. The reduction reaction occurred quickly and proceeded at room temperature for 1 h. The size of TA reduced AgNP was immediately determined by dynamic light scattering (DLS, Malvern) analysis of 1 mg mL\(^{-1}\) of AgNP. Both 2.5 \(\mu\text{L}\) and 5 \(\mu\text{L}\) of the freshly synthesized AgNP (5 mg mL\(^{-1}\)) were each first mixed with 10 \(\mu\text{L}\) of 1 wt% TA solution and then mixed with 30 \(\mu\text{L}\) of 2 wt% HA–PBA solution to fabricate AgNP hybridized dynamic hydrogels. The AgNP concentrations were approximately 2 mM and 4 mM, respectively. Those hydrogels were referred to as the AgNP gels.

2.4 UV-Vis spectra analysis

The UV-visible light absorbance spectra of the AgNP solution, blank gel, and AgNP gel were collected by a microplate reader (BioTek Synergy H1 model) at wavelengths from 400 to 700 nm.

2.5 Scanning electron microscopy and energy-dispersive X-ray spectroscopy (EDX) analysis

The structure and the morphology of the freeze-dried blank and AgNP gels were studied by scanning electron microscopy (SEM,
dish. The dish was covered and kept at 37 °C overnight. They were then freeze-dried in the lyophilizer for 48 h to remove the water thoroughly and transferred to the SEM for examination. For EDX analysis, lyophilized hydrogels were placed on aluminum stubs and adhered with double sided carbon tabs. The stubs were then sputter coated with carbon in a Hummer VI sputter coater from Anatech and viewed in the tungsten gun equipped SEM together with a Bruker AXS Quantax XFlash 4010 EDX detector.

2.6 Rheological studies

The rheological properties of the dynamic hydrogels were characterized at 37 °C with a Discovery HR-2 rheometer (TA Instruments) employing several different protocols. In each study, around 0.2 mL of total hydrogel precursor solution was mixed in an Eppendorf tube and was quickly transferred into the geometry gap between the 20 mm parallel plate and the base plate. Frequency sweeps from 0.1 to 10 Hz were first performed at 10% constant strain to validate the hydrogel formation. The oscillatory amplitude sweep method (γ = 1–1000%), with a fixed angular frequency of 2π rad s⁻¹ (=1 Hz), was carried out to measure the shear storage (G'') and loss moduli (G'') and the critical strain region of dynamic hydrogels. Then, the self-healing characteristics of the hydrogel were determined by the alternate step strain sweep test with a constant frequency (2π rad s⁻¹).

Amplitude oscillatory strains were switched from a small strain (γ = 10%) to a subsequent large strain (γ = 500%) with a 120 s strain interval, and 3 cycles were evaluated. In the end, to evaluate the shear-thinning behavior, flow sweep studies were conducted on the hydrogels with a linearly ramped shear rate from 1 to 50 s⁻¹, and the viscosity was recorded at different rates.

2.7 Self-healing and injectability studies of dynamic hydrogels

Each piece of dynamic hydrogel was prepared by mixing 30 μL of HA–PBA solution, 10 μL of TA solution, and 2.5 μL of food dye solution (red) or 2.5 μL of AgNP solution, as previously mentioned. After gelation, one piece of blank gel was placed close to another piece of dye colored gel or another piece of AgNP gel in a plastic dish. The dish was covered and kept at 37 °C for 60 min to evaluate the self-healing ability. After 60 min, the healed hydrogel was held up and the edges were pulled on with tweezers for observation.

For the injectability test, dynamic hydrogels were loaded into insulin syringes that were capped with a 30-gauge (G) needle to evaluate whether the hydrogel could be extruded through the needle into phosphate-buffered saline (PBS) buffer or star/triangle shaped polydimethylsiloxane (PDMS) molds after manually pushing the syringe plunger.

2.8 Dual stimuli responsive drug release from dynamic hydrogels

Fluorescein isothiocyanate (FITC) labeled bovine serum albumin (FITC-BSA, Sigma) was used as a model drug to evaluate the drug release behavior from dynamic hydrogels. FITC-BSA was added to the pre-dissolved TA solution to prepare 1 wt% TA with 1 wt% FITC-BSA. Dynamic hydrogels were prepared by mixing 30 μL of HA–PBA solution with either 10 μL of TA solution (with FITC-BSA) alone or 10 μL of TA solution (with FITC-BSA) plus 2.5 μL of TA reduced AgNP solution, as previously mentioned. Each hydrogel was individually incubated with 1 mL of PBS alone at pH 5.0 or 7.2 or with 1 mL of PBS at pH 7.2 containing 1 mM H₂O₂ (Fisher Scientific) at 37 °C for 30 h. At every pre-determined time point, the whole release buffer was withdrawn and replaced with 1 mL of fresh buffer. The amount of released FITC-BSA at each time point was determined by fluorescence using a microplate reader (BioTek Synergy H1 model) with the excitation and emission wavelength set at 490 nm and 520 nm, respectively. A standard curve with good linearity (R² = 0.999) was prepared to calculate the released FITC-BSA concentration.

2.9 Cell culture and cytotoxicity study of hydrogel precursor and hydrogel releaseate

The mouse fibroblast cell line-L929 purchased from American Type Culture Collection (ATCC) was used to evaluate the cytotoxicity of the hydrogel precursor and hydrogel releaseate. The L929 cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Invitrogen). For the cytotoxicity study, a total number of 1 × 10⁴ of L929 cells were seeded in each well of a 48-well plate, and the cells were attached on the plate overnight at 37 °C in a humidified incubator (Thermo) containing 5% CO₂. The HA–PBA conjugate of different concentrations from 0.125 mg mL⁻¹ to 1 mg mL⁻¹ and TA of concentrations from 16 to 1000 μg mL⁻¹ were respectively prepared in culture media and added to each well, followed by 24 h incubation. The control group was cultured in the growth media only. To determine the cytotoxicity of the hydrogel releaseate, ~40 μL of each blank gel and AgNP gel (2 mM and 4 mM) were incubated with 1 mL of culture media for 24 h individually. Then the media were collected and added to each well of cells for 24 h culture. Each test group had 4 replicates. The cytotoxicity was determined by an MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay.⁴⁰,⁴¹

2.10 Live and dead assay for cells in contact with dynamic hydrogels

L929 cells were seeded on cover slips in a 6-well plate and cultured until confluency. Dynamic hydrogels (~40 μL) with or without AgNP (2 mM) encapsulation were prepared as mentioned above. Each hydrogel was transferred to one well of the 6-well plate, and 2 mL of cell growth medium was added. L929 cells grown on cover slips in the medium with no hydrogel served as the control. After 24 and 48 h, a live and dead assay was used to test the cell viability by staining the cells on the cover slip with or without hydrogel contact, following our reported method.⁴

2.11 Hemolysis assay

Red blood cells (RBCs) were collected from mouse blood by centrifugation at 2000 rpm for 5 min and washed three times with sterile PBS. The mice were euthanized subjects from another
research protocol, which was approved by the University of Nebraska Medical Center (UNMC) Animal Care and Use Committee. About 200 µL of RBC were dispersed in 12 mL of PBS to obtain an RBC suspension. Each dynamic hydrogel (~40 µL) was immersed individually into 1 mL of RBC suspension, and the mixture was incubated at 37 °C for 1 h. After centrifugation at 3000 rpm for 5 min, the supernatant was collected, and its absorbance at 540 nm was detected using the microplate reader (BioTek Synergy H1 model). PBS and Triton X-100 (1%) were used as negative and positive controls, respectively. Each group had 3 replicates. The percentage of hemolysis in each well was calculated by the following equation:

\[
\text{Hemolysis} \% = \left(\frac{Ab_{\text{sample}} - Ab_{\text{PBS}}}{Ab_{\text{Triton X-100}} - Ab_{\text{PBS}}}\right) \times 100.
\]

### 2.12 Bacterial culture and in vitro antibacterial assay

Two bacterial strains: MRSA USA300 LAC and Pseudomonas aeruginosa (\textit{P. aeruginosa}) PAO1 were obtained from the ATCC.

Disc diffusion study: 100 µL of MRSA or \textit{P. aeruginosa} bacterial suspension with a concentration of \(~10^7\) CFU mL\(^{-1}\) was dripped on a prepared LB agar medium plate and was spread evenly with an L-spreader. After the bacterial liquid was slightly dry, the dynamic hydrogels were placed on the agar plate, then transferred to a 37 °C incubator for 12 hours. The diameter of the inhibition zones was measured, respectively.

The minimum inhibitory concentrations (MIC) assay of our TA reduced AgNP were performed against \textit{P. aeruginosa} and MRSA by using a broth microdilution protocol. \(^{42}\) In short, 100 µL of TA reduced AgNP were added to each well of a 96-well microplate at different concentrations (0.5–1000 µg per mL). Another 100 µL of 10^6 CFU mL\(^{-1}\) bacteria suspensions were then added to the wells to allow the final microbial concentration to be 5 × 10^5 CFU mL\(^{-1}\). The plate was placed in an incubator at 37 °C for 18 h. The MIC was determined to be the lowest concentration, with no visible microbial growth detected with a microplate reader at 600 nm (BioTek Synergy H1 model).

Bacterial suspension assay: 400 µL of MRSA or \textit{P. aeruginosa} bacterial suspension with \(~5 \times 10^5\) CFU mL\(^{-1}\) concentration were added to each well in a 48-well plate. Dynamic hydrogels with and without AgNP were added as test samples. The plates were wrapped in aluminum foil and placed in a shaker at 37 °C for 18 h. The OD readings were collected by measuring the absorbance at 600 nm using a Nanodrop spectrophotometer (Thermo). Each assay was performed in triplicate.

### 2.13 Antibacterial activity of dynamic hydrogels coated on a glass slide

Dynamic hydrogels were injected through syringes and evenly spread on 10 mm cover slips. Each cover slip, with or without coating, was transferred into a well of a 12-well plate. For the antibacterial assay, a \textit{P. aeruginosa} suspension (0.5 mL, \(5 \times 10^5\) CFU mL\(^{-1}\)) was added onto the cover slips coated with or without dynamic hydrogels in each well. After incubation at 37 °C for 24 h, the cover slips were removed from the well and gently washed with PBS. Then the bacteria grown on cover slips were stained with the live and dead assay and imaged under confocal microscopy.

### 2.14 DPPH free radical scavenging assay of dynamic hydrogels

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate, Alfa Aesar) free radical scavenging capacity of dynamic hydrogels was investigated following a reported method. \(^{44}\) In brief, 0.1 mM DPPH solution was prepared in ethanol, and 0.5 mL of freshly prepared solution was added to 1.5 mL of ethanol (as control) or ethanol containing either tannic acid (100 µg), HA–PBA conjugate (600 µg), or the dynamic hydrogel (~40 µL). These solutions were vortexed thoroughly and incubated in the dark for half an hour. The absorbance was measured at 517 nm against blank samples by a UV spectrometer (WPA Biowave II+). Each group had 4 replicates. The DPPH free radical scavenging effect was calculated using the equation:

\[
\text{Scavenging effect} \% = \left[1 - \frac{Ab_{\text{sample}}}{Ab_{\text{control}}}\right] \times 100
\]

### 2.15 ROS/hydrogen peroxide reactive fluorescent staining

To investigate the hydrogen peroxide (H_2O_2) scavenging effect of dynamic hydrogels, an H_2O_2 reactive fluorescent probe 2',7'-dichlorodihydro fluorescein diacetate (H_2DCFDA, Millipore) was used to test the intracellular H_2O_2 levels after H_2O_2 treatment. \(^{4} \) L929 cells were seeded in each well (\(1 \times 10^5\) per well) of a 24-well plate and were attached to the plate overnight. Four pieces of AgNP hydrogels (~40 µL) were individually added into four wells, and the cells in each well, with or without the hydrogel addition, were cultured in H_2O_2 (0.4 mM) containing growth medium for 2 h. The H_2DCFDA probe was prepared as a 5 mM stock solution in DMSO and diluted to 5 µM with sterile PBS before use. The hydrogel and the H_2O_2 containing medium were removed, and cells were incubated in a 5 µM probe solution containing DRAQ-5 (Cell Signaling) as a nuclei marker for 20 min at 37°C before microplate reading and confocal microscopy (Zeiss 880) imaging. Cells cultured in the growth medium alone served as a control. The intracellular fluorescence was excited at 480 nm and emitted at 540 nm.

### 2.16 Statistical analysis

Quantitative data is expressed as the mean ± standard deviation (SD). The statistical analysis was carried out using student t-tests in GraphPad Prism 7.0 Software. A p-value less than 0.05 was considered statistically significant. In all cases, * represents \(p < 0.05\), ** represents \(p < 0.01\) and *** represents \(p < 0.005\).

### 3. Results

#### 3.1 Fabrication and characterization of dynamic hydrogels

The full steps for preparing the dynamic hydrogel were started with the synthesis of a HA–PBA conjugate by grafting the PBA moiety to the HA backbone via an amide bond. EDC/HOBt was used as the coupling agent. The schematic synthesis of HA–PBA is shown in Fig. 1A. NMR analysis confirmed a high efficiency conjugation, and we could consistently achieve a >95%
substitution degree of PBA moiety in the HA backbone (Fig. S1, ESI†). The HA–PBA–TA dynamic hydrogel was facilely prepared by mixing an HA–PBA solution with a TA solution at room temperature, and the gelation occurred rapidly (<30 s) via the complexation between the phenylboronic acid group from HA–PBA and the gallate/catechol groups from TA (Fig. 1B). TA is also a good reducing agent and provides a simple, quick, and efficient method for the green synthesis of AgNP here (Fig. 1B).35 After adjusting the pH of TA solution to neutral, Ag⁺ ions were easily reduced to silver nanoparticles of sizes ranging from 10 to 35 nm with a median size of 15 nm (Fig. 2A), as determined by the DLS. The dual functions of TA were combined to prepare the AgNP encapsulated dynamic hydrogels. The stepwise preparation could result in a homogeneous dispersion of AgNP in the dynamic hydrogel. The HA–PBA–TA dynamic hydrogel itself was colorless but became brown with the dispersed AgNP (Fig. 2B). The UV-visible light absorbance spectra of the blank and AgNP gels were scanned, and the characteristic absorption peak at 410 nm confirmed the presence of AgNP inside hydrogel (Fig. 2C). The encapsulated AgNP concentration was adjustable without affecting the dynamic hydrogel formation, and when the AgNP concentration increased, the gel turned into darker color (Fig. 2B). SEM of the lyophilized samples showed microporous structures and revealed that the incorporation of AgNP in the dynamic hydrogel had no remarkable effect on the pore size and morphology of the dynamic hydrogel (Fig. 2D). EDX mapping further confirmed the incorporation of AgNP within the hydrogel (Fig. S2, ESI†).

The rheological properties of the dynamic hydrogels were thoroughly investigated. Frequency sweep testing of both hydrogels indicated that the $G''$ was constantly lower than the $G'$ over the tested frequency range, showing a good maintenance of their elastic characteristics for these dynamic hydrogels. The storage modulus ($G'$) of the blank and AgNP gels (2 mM) reached $317 \pm 7$ Pa and $425 \pm 19$ Pa at a moderate 10% strain. The slightly smaller $G'$ value of the AgNP gel was presumably attributed to the dilution of the TA concentration after the incorporation of the AgNP solution at the beginning. The intersection, or critical point, appeared at nearly 450% for the blank gel and about 500% for the AgNP gel (Fig. 3B). When the strain was continuously increased, the $G'$ value was dramatically decreased and became less than the $G''$ value, implying a collapse of the hydrogel network at a large strain ($\geq 500\%$).

The self-healing property of the dynamic hydrogel is a result from the dynamic boronic ester bond. It was first evaluated by the rheological recovery test (Fig. 3C). The recovery process was consistent during the three cycles of alternate step strain of 10% and 500%. When the hydrogel was subjected to 500% strain, the $G'$ value immediately decreased from 315 Pa to 93 Pa for the blank gel and from 421 Pa to 130 Pa for the AgNP gel. Once the strain returned to the initial low strain, the $G'$ value quickly recovered to the initial $G'$ value for both dynamic hydrogels.

**Fig. 2** Characterization of the HA–PBA–TA dynamic hydrogel loaded with AgNP. (A) DLS results of AgNP at 1 mg mL⁻¹ showed a median size of 15 nm with the size range between 10–35 nm; (B) appearance of the dynamic hydrogel without AgNP (blank gel) and with different concentrations of AgNP (AgNP gel); (C) UV-visible light spectra of AgNP solution, blank hydrogel, and AgNP hydrogel; (D) SEM images of dehydrated blank gel and AgNP gel (2 mM).
The injectability of the hydrogel was shown by the acute decrease of the viscosity with increasing the applied shear rate (Fig. 3D). The viscosities for the blank and AgNP gels were 286.4 and 175.1 Pa s, respectively, at a shear rate of 1.25 s⁻¹ and substantially declined to 25.3 and 20.1 at the rate of 50 s⁻¹ for the blank and AgNP gels, respectively.

To better prove the self-healing property, after rheometer testing, dynamic hydrogels were cut in half. Then the two pieces were put together for healing for 10 min, and the rheological properties were measured again. It was found that the storage modulus of the healed hydrogel easily recovered to nearly 91% and 95% of the level before cutting (Fig. S3, ESI†) for the blank and AgNP gels, respectively.

The injectability and self-healing properties of two hydrogels were also examined macroscopically. Both the blank gel and the AgNP gel (2 mM) were loaded into insulin syringes and could be easily extruded through the 30 G needle as fluids into a PBS buffer or shaped molds (Fig. 4A and B), where they returned to the gel state again. A blank gel was placed either next to a red dye colored hydrogel or in contact with the AgNP gel, allowing them to heal with each other at 37 °C for up to 60 min. It was noticed that the boundaries between the contacting gels gradually became obscure. The red dye from the other piece of gel diffused into the blank gel, however, the yellow color (or AgNP) did not diffuse into the blank gel in such a short time, possibly due to the relatively slower diffusion rate of AgNP inside the gel. In both conditions, the two pieces of hydrogels were healed together as a single hydrogel and could be easily lifted by holding one end of the hydrogel. The pulling of the healed hydrogel on both ends did not cause any breakage on the healing zone (Fig. S4A, ESI†), and it was further demonstrated by the tensile stress curve of the dynamic hydrogel before cutting and after healing (Fig. S4B, ESI†). These results, combined with the rheological recovery results, indicated good self-healing properties of the hydrogels.

3.2 pH and ROS responsive drug release from the dynamic hydrogels

To determine whether the dynamic hydrogel could serve as a drug delivery system, we investigated the release of FITC-BSA from the HA–PBA–TA hydrogel under pH 7.2 and pH 5.0, which mimic the pH of chronic wounds and healthy skin, respectively. The release of BSA under pH 7.2 with 1 mM H₂O₂ was also evaluated to mimic both the increased ROS and pH in chronic wounds. As shown in Fig. 5A, only about 3% of FITC-BSA in the blank gel was released after 30 h when the gel was immersed in a pH 5.0 buffer, while the value markedly increased to 88% when immersed in a pH 7.2 buffer. More importantly, under buffer pH 7.2 with 1 mM H₂O₂ conditions, BSA was completely released within 20 h, and the hydrogel was almost degraded (Fig. 5A and Fig. S5, ESI†). Similarly, the AgNP gel (2 mM) also showed the pH and H₂O₂ responsive release profile from the gel (3% at pH 5.0 and 92% at pH 7.2 at 30 h; 100% at pH 7.2 with 1 mM H₂O₂ at 20 h, Fig. 5B). The pH responsive release of the FITC-BSA from the gel was probably caused by the switch between phenolic hydroxyls and quinones of TA tuned by the environmental pH.34 TA has a central core of glucose and is rich with phenolic hydroxyl groups (9–10 gallic acid residue), which form strong hydrogen bonds with the BSA molecules.44 When the solution’s pH was adjusted to neutral or slightly basic (7.0–7.4), the phenolic hydroxyl would transfer into quinone groups, which resulted in decreased interaction of TA with the BSA molecule as well as the phenylboronic acid group. Both should lead to accelerated drug release from the hydrogel. In the presence of
H$_2$O$_2$, the degradation of boronic ester together with the oxidation of TA into quinone forms caused an enhanced erosion of the hydrogel network, accompanied with the fastest release of BSA from the hydrogel.

### 3.3 Cytotoxicity and hemolysis assay

We investigated the cytocompatibility of the dynamic hydrogels and their components. L929 cells could maintain over 90% viability when incubated with as high as 1 mg mL$^{-1}$ HA–PBA conjugate (Fig. 6A), suggesting low toxicity of the synthesized polymer conjugate. TA, as a natural polyphenol, shows minimal toxicity at concentrations up to 250 mg mL$^{-1}$, but significantly reduced the cell viability at concentrations of 500 mg mL$^{-1}$ or higher (Fig. 6B). Both the blank and the AgNP gels (2 mM) were biocompatible, as shown by the hydrogel releasate cytotoxicity results and the live and dead fluorescence staining of cells incubated with the gels (Fig. 6C–E). However, higher concentrations of AgNP encapsulated in the gel caused lower cell viability ($p < 0.005$) (Fig. 6C), which might limit its in vivo application due to safety concerns. The degree of hemolysis is another important factor in evaluating the biocompatibility of biomaterials that can directly contact red blood cells during application (i.e. wound dressing and hemostatic agents). The hemolytic toxicity of two hydrogels was evaluated by measuring the hemolytic potential for RBCs. Only 1% of RBCs were lysed after incubation with the blank gel; although the hemolysis ratio for the AgNP gel (2 mM) was significantly larger than the blank gel, probably arising from the initial released AgNP, it was still less than 10% (Fig. 6F and Fig. S6, ESI†) and was considered nonhemolytic according to the in vitro hemolysis.
guidance, confirming limited hemolytic toxicity of the dynamic hydrogels.

3.4 *In vitro* antibacterial activities

The disc diffusion assay was first used to test the antimicrobial properties of the blank and AgNP gels (2 mM) against both Gram-negative bacteria (*i.e.*, *P. aeruginosa*) and Gram-positive bacteria (*i.e.*, MRSA). As expected, evident zones of inhibition were only observed around the AgNP gels but not the blank gels (Fig. 7A), indicating that the antibacterial activity was from the presence of silver nanoparticles in the AgNP gels. It seems that Gram-negative bacteria was more sensitive to the AgNP gel than the Gram-positive, as shown by the zone of inhibition results (Fig. 7B), which is correlated with the MIC values. This might be because the Gram-positive bacteria have a plasma membranes covered with a thick layer of peptidoglycan, which could restrict the penetration of the AgNP. To better evaluate the bacterial killing ability of the AgNP gel, suspension assays were conducted by measuring the optical density (OD) of bacteria at 600 nm. The results show that for both bacterial strains, the OD values decreased remarkably for the AgNP gel, in comparison to the control and blank gel (Fig. 7C). Those results, and the fact that only few colonies were proliferated in the LB agar inoculated with bacterial suspensions that were incubated with AgNP gel (Fig. S7, ESI†), demonstrate that the AgNP gel can not only inhibit bacterial growth but also kill them.

The AgNP gel (2 mM) was also coated on glass cover slips to evaluate its antibacterial activity as a coating (Fig. 7D). The glass surface with or without AgNP gel coatings was incubated with a PA suspension and then stained by the live and dead assay. It was found that the PA were effectively killed when incubated on the AgNP gel coated cover slips. As shown in Fig. 7D, the uncoated surface incubated with PA was stained with strong green fluorescence due to bacterial adherence and proliferation, while the AgNP gel coated surface showed almost no fluorescence, suggesting efficient killing of the bacteria. In contrast, the blank gel coated
surface also showed fluorescence as strong as the uncoated surface, implying the important role of AgNP in the AgNP gel. These results indicated that the AgNP gel could be potentially used as coatings for local antibacterial applications.

3.5 In vitro anti-oxidative property

The anti-oxidative property was first evaluated using the DPPH free radical scavenging assay. Both the blank and AgNP gels (2 mM) showed high free radical scavenging capabilities, comparable to the equivalent amount of TA, while HA–PBA itself had minimal DPPH free radical scavenging effects (Fig. 8A), revealing that the scavenging effect mostly resulted from the TA part instead of the HA–PBA conjugate. H₂O₂ is an important ROS generated in chronic wounds. 47 Both the boronic ester and TA have been found to have H₂O₂ scavenging capabilities. 4,16 To better confirm the H₂O₂ scavenging ability of the dynamic hydrogel, the cells were treated with H₂O₂ in the medium, and the intracellular H₂O₂ level was measured by an H₂DCFDA probe. H₂DCFDA is cell permeable and non-fluorescent, but it converts into a fluorescent product when activated by intracellular H₂O₂. 4 Both microplate fluorescence readings and confocal microscopy imaging results revealed that the intracellular H₂O₂ levels were elevated when cells were incubated in H₂O₂ containing media; however, when AgNP dynamic hydrogels were added, the intracellular H₂O₂ levels were significantly decreased (p < 0.005) (Fig. 8B and C).

4. Discussion

Chronic wounds, such as foot ulcers, are the leading cause of hospitalization and limb amputation in patients with diabetes and generate a great economic and healthcare burden on society. 48 Unfortunately, in the past decade, there has been very limited breakthrough in the clinical treatment strategy for chronic wounds. Bioactive hydrogels are potential wound dressing candidates for aiding the closure of chronic wounds, and hydrogels based on dynamic bonds provide a better choice due to their injectability and self-healing characteristics. Among diverse dynamic bonds, the boronic ester dynamic covalent bond has been exploited for many biomedical applications, such as analyte sensors, disease diagnosis, and drug delivery, due to its distinguishing multi-stimuli (pH, glucose and H₂O₂) responsiveness. 23–25 Most phenylboronic acid derivatives have low toxicity, and engineered hydrogels based on boronic ester have been developed. 21 The formation of boronic ester bond cross-linked hydrogels normally requires alkaline pH conditions, but very few studies have been reported to prepare such type of dynamic hydrogels at an acidic pH. 49 In this study, we developed a multifunctional HA–PBA–TA dynamic hydrogel that could form under an acidic pH by simply mixing the solution of synthesized HA–PBA conjugate with the acidic TA solution without pH adjustment, which was expected to increase the stability of TA and prevent its precipitation. 50 The boronic ester formation at the acidic pH was attributed to the intramolecular coordination between the carbonyl oxygen of the amide and the boron of the boronic acid group when 2-aminophenyl boronic acid was conjugated via an amide to the HA backbone, which helped stabilize the tetrahedral form required for strong interactions between boronic acid and diols/catechols. 49 In contrast, another HA–PBA that was synthesized using the 3-aminophenyl boronic acid molecule, which lacks the intramolecular coordination, was only able to complex with TA to form nanoparticles but not a hydrogel. The resultant nanoparticles showed higher antibacterial effects than TA itself, but it easily hydrolyzed in an acidic pH (~5), which
could hamper its biomedical applications.\textsuperscript{51} However, no similar hydrogels have been developed so far. By implementing the dynamic boronic ester bond, hydrogels were easily endowed with shear-thinning and self-healing properties (Fig. 4C, D and 5C, D), which should help inject them easily into the wound area using syringes (Fig. 4A and Fig. S4C, ESI\textsuperscript{†}) and maintain their structural integrity during the wound healing process.

Although the natural product, TA, has been utilized to fabricate antibacterial hydrogels by taking advantage of the inherent antibacterial activity of TA,\textsuperscript{32} the toxicity of TA toward mammalian cells cannot be overlooked. The toxicity concern has caused the abandoning of the use of TA in burn wound therapy since the 1940s.\textsuperscript{52} Based on our results, TA at 0.5–1 mg mL\textsuperscript{-1} in solution greatly reduced mouse fibroblast viability (Fig. 6B). So, it is better to construct the hydrogel with TA at a low concentration.\textsuperscript{16} In our case, TA as low as 0.25 wt% was enough to be able to fabricate hydrogels with a proper viscoelasticity that is fit for the wound dressings. This is advantageous when compared to two disclosed hydrogel systems based on boronic ester comprised TA, which contained at least 20 wt% and 2 wt% of TA, respectively.\textsuperscript{26,53} In addition, at the healthy skin pH (\textasciitilde 5), the dynamic hydrogel network was stable enough to release minimal free TA, which would further reduce the potential toxicity of our hydrogel containing TA.

To enhance the antibacterial activity of the dynamic hydrogel with a low amount of TA, the catechol redox of TA was exploited and resulted in the green synthesis of AgNP, which was then homogeneously distributed in the hydrogel network to develop a dynamic hydrogel proven to have potent and broad-spectrum anti-bacterial activity (Fig. 7). The size of AgNP can be finely tuned in a wide range of 7–200 nm by varying the reaction pH as well as the Ag\textsuperscript{+} and TA concentrations.\textsuperscript{54} The antimicrobial activity of TA reduced AgNP was dependent on the size of the nanoparticles.\textsuperscript{36} TA reduced AgNP have displayed enhanced anti-bacterial and anti-biofilm activities as compared to unmodified AgNP.\textsuperscript{36,37} The MIC value of a reported TA reduced AgNP of 13 nm was 3 \textmu g mL\textsuperscript{-1} towards PA,\textsuperscript{16} which was close to our determined value (Fig. 7B). Our results validated that the TA reduced AgNP could be facilely encapsulated in the hydrogel matrix and preserved their strong bactericidal activity, which potentiated the biomedical application of TA reduced AgNP. The potential anti-biofilm activity was also validated with the AgNP hybridized dynamic hydrogel, as we found biofilm-like membrane formation in both the non-coated and blank gel coated glass when incubated with PA suspensions but no such membrane in the AgNP gel coated glass. Confocal imaging indicated a mass of live bacterial in the membranes from the blank gel and control samples but very few live bacterial from the AgNP gel, which also confirmed the anti-biofilm ability (Fig. 7D).

In summary, our work proved that the dual functions of TA as both the hydrogel precursor and a reducing agent could be successfully combined to develop a green and handy process
to prepare injectable hydrogels with strong antimicrobial properties. This strategy is also much convenient as compared to other in situ synthesized AgNP hybridized hydrogels requiring the introduction of additional catechol groups into the hydrogel precursor.\textsuperscript{55}

It is known that the wound healing course is governed by the spatio-temporal abundance of certain growth factors in the wound site at different stages.\textsuperscript{56} However, the variations of pH and oxidative stress in chronic wounds have been found to indirectly cause the degradation of some important growth factors, such as VEGF, which contributes to disorganized wound healing.\textsuperscript{57–59} It will be beneficial if the injectable hydrogel used as the growth factor delivery system could modulate the growth factor release rate in response to the local pH or ROS, thereby achieving appropriate doses within different stages to restore wound healing.\textsuperscript{60} Recently, a wound pH sensitive VEGF delivery system was achieved by a poly(N-isopropylacrylamide-co-acrylic acid)-based thermosensitive hydrogel and proved to have better wound healing efficacy in comparison to a wound pH insensitive VEGF delivery system comprised of a poly(lactide-co-glycolide) microsphere.\textsuperscript{60} TA could form a complex with proteins, such as BSA, and the complex was easily dissociated under an alkaline pH.\textsuperscript{61,62} The pH dependent protein binding ability of TA was employed in our dynamic hydrogel to carry protein-based drugs or growth factors. The release of the model protein, BSA, from the dynamic hydrogels was dependent on the pH and ROS (Fig. 5). In chronic wounds, both a pH increase and an elevated ROS level will result in enhanced protein release. VEGF has shown beneficial effects in foot ulcer treatment.\textsuperscript{62,63} When the VEGF is loaded, the dynamic hydrogel could promote the wound healing by promoting chemotaxis and angiogenesis with the rapid release of VEGF.\textsuperscript{64} With the killing of bacteria and the wound getting healing, the wound pH shifts back to an acidic condition, and the release of VEGF from the hydrogel is expected to decrease, which may reduce scar formation.\textsuperscript{65} However, it remains to be validated that VEGF activity would not alter after binding and then cause dissociation from TA and release from the hydrogel.\textsuperscript{66} 

In healthy individuals, the increase of the right amount of ROS is helpful and accelerates the wound healing, as ROS at low concentrations are important regulators of diverse gene expression.\textsuperscript{15} However, in patients with diabetes, excessive ROS (particularly O$_2^-$ and H$_2$O$_2$) caused by a redox imbalance have been shown to hinder wound healing through many pathways, including inactivating growth factors and reducing extracellular matrix production and deposition.\textsuperscript{14} Anti-oxidative strategies have been proven to effectively accelerate the chronic wound healing in animal models after treatment with different antioxidants from small molecules to relatively large macromolecules and nanoparticles.\textsuperscript{19,66,67} Anti-oxidative hydrogels prepared through either the incorporation of antioxidants in the polymer network or the use of an ROS-responsive hydrogel precursor/cross-linker have also shown efficacy toward diabetic wound healing in mice.\textsuperscript{68–70} However, until recently, people have realized the importance that both the infection and ROS imbalance should be synchronically targeted in chronic wounds to achieve synergistic effects for wound regeneration.\textsuperscript{13,69} Studies have elucidated that, for one, without high ROS, biofilms collected from old chronic wounds did not create chronic wounds in new excision sites; for another, high ROS did not cause chronic wounds in the absence of bacterial infection.\textsuperscript{71} Our AgNP gel provides a convenient agent to target both of them, and future studies will focus on evaluating the in vivo efficacy for treating infection and promoting wound healing using the AgNP dynamic hydrogel system.

### 5. Conclusions

In summary, we have facilely synthesized dynamic hydrogels with self-healing and shear-thinning properties caused by the rapid dynamic exchange of the boronic ester bond. The hydrogel could be in situ hybridized with TA reduced AgNP and presents potent and broad-spectrum bactericidal properties. We further demonstrated that the injectable hydrogels could be applied for pH and ROS responsive drug delivery to target chronic wound microenvironment changes. The hydrogels were cytocompatible with limited hemolytic activity. The AgNP hybridized hydrogels could also be used as coatings on medical devices/implants to prevent bacterial growth. Moreover, because of the intrinsic antioxidative activity of the hydrogel precursors, the dynamic hydrogels displayed ROS-scavenging capabilities. The multifunctional dynamic hydrogels are expected to promote chronic wound healing, and further in vivo animal studies will be required to demonstrate the efficacy in the future.

### Conflicts of interest

There are no conflicts of interest to declare.

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### References


