Puerarin-containing Rhein-crosslinked Hydrogel for Antibacterial and Anti-Inflammatory Wound Dressings

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Abstract

Wound infections put patients at risk for serious physical harm, even death, and place a heavy financial strain on society. As such, they are one of the most pressing issues facing society today. There is an immediate need for hydrogel dressings with immune-regulating and antibacterial properties. This work is predicated on the two small natural molecules’ complementary physical and efficacious properties. An injectable hydrogel is created by loading the puerarin (PUE) into tyramine-modified hyaluronic acid, then adding rhei (RHE) and \( \text{H}_2\text{O}_2/\text{horseradish peroxidase} \) to facilitate the formation of inter-phenol crosslinks (HA-Tyr-R@P). With its ability to release oxygen and scavenge active oxygen, the developed hydrogel exhibits excellent biocompatibility and modifies the microenvironment favorably. HA-TYR-R@P hydrogels have an antibacterial rate of over 95% and a wound closure rate twice as high as the control group. HA-TYR-R@P hydrogels can inhibit bacteria, reduce inflammation in wounds, encourage wound healing, and have good biocompatibility because of their matched physicochemical characteristics and synergistic therapeutic effects. This work emphasizes the creation of a single-component, multipurpose hydrogel platform that eliminates the need for complex preparations and side effects associated with drugs. Additionally, it provides a comprehensive approach for creating RHE-based hydrogel for wound healing and highlights the importance of natural small molecules in the advancement of biological materials.

1. Introduction

The skin is the largest and most external organ in the human body and, among other things, holds the task of maintaining the stability of the internal environment and preventing the penetration of pathogens\(^{[1, 2]}\). Normally, the skin has a certain ability to heal itself after damage. However, if the wound heals with difficulty or the healing time is prolonged, it is easily contaminated by complex groups of bacteria from various sources (external environment, surrounding skin and endogenous), which greatly increases the risk of infection and can lead to death in severe cases. Bacteria have the ability to proliferate in chronic wounds, leading to the development of a wound infection\(^{[3]}\). Statistics indicate that germs are prevalent in chronic wounds, which significantly contributes to the delayed healing process. The process of wound healing is intricate. The process has three primary interconnected phases: inflammation, generation, and restructuring of fresh tissue\(^{[4]}\). The immune response during the initial phases of wound healing is vital for the process of tissue regeneration\(^{[5]}\). Although inflammation at tissue damaged sites is necessary to trigger a healing response, elimination of inflammation is also important to promote the healing process and restore tissue integrity\(^{[6, 7]}\). Macrophages have a significant role in tissue regeneration and wound healing. Fibroblasts play a crucial role as the primary producers of cytokines during the wound healing process. They exert control over this process by releasing cytokines. The majority of the initial adhering macrophages exhibit M1 differentiation, which leads to the release of diverse inflammatory cytokines, subsequently triggering inflammation and eliminating microorganisms. Macrophages undergo development into M2 phenotypic cells, which suppress inflammation and facilitate the differentiation of stem cells, as well as promote tissue repair and regeneration\(^{[8, 9]}\). During the healing process of infected chronic wounds, it plays a key role in skin repair by regulating the
polarization and phenotypic immune response of macrophages. Empirical research demonstrates that effective control of the equilibrium between pro-inflammatory (M1) and anti-inflammatory (M2) factors facilitates the process of wound healing\cite{8, 10, 11}. Thus, the optimal wound dressing should be antibacterial to prevent M1 macrophage hyperactivation and should be able to modulate the M1/M2 ratio to control the immune response, thus facilitating the healing process of infected wounds.

The exceptional biocompatibility, adaptable ingredient design, and uncomplicated preparation methods of hydrogels derived from natural components have garnered significant interest for their application in the therapy of injured tissue. Multiple studies have demonstrated that plant-derived natural small molecules have a significant impact in inhibiting bacterial growth\cite{12}. Examples of such molecules include rhein (RHE)\cite{13}, berberine\cite{14}, curcumin\cite{15}, etc. RHE, or Rhein, is the primary component found in various traditional Chinese remedies like Rhubarb, Aloe, and Sennae Folium. It possesses numerous pharmacological actions, including anti-inflammatory, anti-tumor, anti-fibrotic, and antioxidant properties\cite{16–19}. Nevertheless, its therapeutic usage is restricted because to its inadequate water solubility and limited absorption\cite{20}. Recent studies\cite{21} have revealed that RHE can easily form a stacking structure in solution due to the unique anthraquinone framework, hydrogen bonding, pp interaction and hydrophobic effect and thus be directly assembled into a hydrogel. In addition, researchers have also developed Rhine-based hydrogels for the treatment of skin wounds and achieved certain therapeutic results\cite{13, 21}. However, antibacterial hydrogels can hardly meet the immunomodulation needs of infected chronic wounds. Hence, there is a pressing need to enhance the immunoregulatory properties of RHE-based hydrogel in order to promote wound healing.

Wound infections are the primary factor responsible for impeding wound healing\cite{7, 22}. Lipopolysaccharide (LPS) released by bacteria is a classic factor that induces the activation of macrophage M1\cite{23, 24}. Therefore, the antibacterial effect and regulation of the stable state of the inflammatory response microenvironment are crucial to ensure wound healing. By introducing anti-inflammatory agents based on small natural molecules, the immune regulation ability of rhubarb acid-based hydrogels can be achieved. PUE, derived from Pueraria, possesses the anti-inflammatory, antioxidant defense and oxidative stress-relieving properties of Pueraria\cite{25–27}, and is used in the treatment of myocardial infarction and diabetes\cite{28}. This work is predicated on the two small natural molecules’ complementary physical and efficacious properties. An injectable hydrogel is created by loading the puerarin (PUE) into tyramine-modified hyaluronic acid, then adding rhein (RHE) and H₂O₂/horseradish peroxidase to facilitate the formation of inter-phenol crosslinks (HA-Tyr-R@P) (Scheme 1)\cite{29}. In combination with a variety of rheological tests, the HA-TYR-R@P hydrogel shows good self-healing, is thixotropic and injectable and is suitable as a wound dressing. Specifically, the HA-TYR-R@P hydrogel that has been produced has superior antibacterial properties compared to its administered medication, while also demonstrating little cytotoxicity and hemolytic toxicity. We have found that the HA-TYR-R@P hydrogel effectively reduces the inflammatory response generated by bacterial toxic substances. This confirms that the hydrogel promotes the healing of infected wounds in the body and
demonstrates good biocompatibility. Therefore, this HA-TYR-R@P hydrogel with antibacterial activity and immunomodulation synergy serves the purpose of promoting the healing of infected chronic wounds.

2. Methods

2.1. Materials

Hyaluronic acid (HA, Macklin, China), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC, Macklin, China), N-hydroxsuccinimide (NHS, Rhawn, China), tyramine (Tyr, Macklin, China), horseradish peroxidase (HRP, Solarbio, China) were used in the preparation of HA-TYR-R@P hydrogel. Reagents used for the biological experiments included Fetal bovine serum (FBS; Tianhang, China), Roswell Park Memorial Institute 1640 (RPMI, Gibco), Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco), phosphate-buffered saline pH 7.2 (PBS, Servicebio, China), and CCK-8 (Biosharp, China) reagent. Without any additional purification, all of the analytically graded chemicals were utilized.

2.2. Synthesis of HA-Tyr

A solution of HA was prepared by dissolving 1 g of HA in 100 mL of deionized water. Subsequently, 2.3 g of NHS and 3.83 g of EDC were introduced into the mixture. The resulting solution was then agitated for a period of one hour to initiate the activation of the carboxyl group. After adding 2.62 g of Tyr, the mixture underwent a 72-hour reaction at room temperature. Regenerated cellulose dialysis tubing with a molecular weight cut-off (MWCO) of 3.5 kDa was employed to perform dialysis on the raw product solution in water for a duration of 3 days. Subsequently, the polymer solution underwent lyophilization.

2.3. Preparation of HA-Tyr-R hydrogel

NaHCO₃ (pH 8.3, 0.2 M) was used to dissolve HA-Tyr polymer at a concentration of 3%(w/v). Heating the HA-Tyr solution caused varying amounts of rhein to dissolve. The freshly made solutions of hydrogen peroxide (H₂O₂, 50 mmol/L) and HRP (50 U/mL) were combined with the HA-Tyr solution, which contained varying amounts of rhein, to create the HA-Tyr-R hydrogels.

2.4. Characterizations

Samples underwent a 1H NMR analysis using D₂O as the solvent on an AVANCE III, 400MHz, Bruker nuclear magnetic resonance (NMR) spectrometer.

2.5. Rheology Test

Using an 8-mm parallel-plate rheometer (MCR302, Anton Paar), the rheological characteristics of the bulk HA-Tyr-R hydrogels were assessed. The time sweep was carried out with a fixed strain (0.1%) and frequency (1 Hz) over a range of time intervals from 0 to 10 min. By setting a 0.1% strain and adjusting the frequency within a range of 0.01 to 10 Hz, a frequency sweep was completed. There were two steps involved in the step-strain rheological experiment. The HA-Tyr-R hydrogels were first tested using a 0.1% strain (step 1). Next, in order to fully destroy the gels, the strain was increased from 0 to 300 and
maintained there for 100 s (step 2). The composite viscosity was determined by measuring its response to angular frequencies ranging from 0 to 630 throughout a test duration of 700 seconds.

### 2.6. Antibacterial Activity of HA-TYR-R@P Hydrogel In Vitro

In order to assess the antibacterial effectiveness of each sample in a controlled laboratory setting, the Gram-positive bacterium Staphylococcus aureus and the Gram-negative bacterium Escherichia coli were employed. Prior to each experiment, the bacteria were cultivated on Lucia bertani (LB) plates for a whole night at a temperature of 37°C. Every individual strain of bacteria was isolated into a single colony, which was subsequently grown in 4 mL of LB medium at a temperature of 37°C for the whole time. The bacteria were diluted to a concentration of 106 colony forming units (CFUs) per milliliter for the remaining tests after undergoing two rounds of cleaning with sterile PBS (10 × 10^{-3} m, pH 7.4). The hydrogel that had been created in a 24-well plate for 12 h was disinfected using UV light. Following the addition of 500 µL of a prepared bacterial suspension (1 × 10^{6} CFUs·mL^{-1}) to each well, the samples were incubated at 37°C for different time intervals (1, 3, 6, and 8 h). The spread plate technique (SPM) was subsequently utilized to quantify the antibacterial efficacy of each sample.

### 2.7. Live/Dead Bacterial Staining Analysis

A UV light was used for 12 hours to disinfect the hydrogel that had formed in the 24-well plate. For six hours at 37°C, 500 µL of a prepared bacterial suspension (1 × 10^{6} CFUs·mL^{-1}) was added to each well. The bacteria suspensions were subjected to hydrogel treatments, followed by three gentle PBS rinses, and 30 minutes of darkness were required for the staining of 500 µL staining reagent mixtures, as per the illustration provided by Invitrogen for their Live/Dead BacLight bacteria viability kits. Lastly, the Leica DMI8, a German fluorescence microscope, was used to view the bacterial suspension. Red fluorescents were thought to represent dead bacteria, while green fluorescents were thought to represent living bacteria.

### 2.7. Cell Culture and Cytotoxicity of HA-TYR-R@P Hydrogels

Sichuan University supplied mouse mononuclear macrophage leukemia cells RAW264.7 and mouse fibroblast cell line L929. RAW264.7 cells were cultured in DMEM supplemented with 15% FBS and 1% antibiotic-antimycotic-amphotericin. On the other hand, L929 cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin and streptomycin-amphotericin. The cultures of both groups were kept at a temperature of 37°C in a humid environment with a CO_{2} concentration of 5%. The biosafety of HA-TYR-R@P hydrogels was assessed using a CCK8 kit and a LIVE/DEAD staining kit. Specifically, a volume of 1 mL containing L929 cells was placed into a 24-well plate with a concentration of 2.0 × 10^{4} cells·mL^{-1}. The cells were then incubated for a period of 12 hours. Subsequently, a Transwell holding 0.5 mL of each sample was meticulously introduced to the plate, and it was allowed to undergo culture for a further 24 and 48 hours. Following the addition of 100 µL of CCK8 solution and a two-hour incubation period, the absorbance at 450 nm was measured using a microplate reader. LIVE/DEAD cell staining was used to assess the biocompatibility of HA-TYR-R@P hydrogels in more detail. The procedure
involved seeding 1 mL of L929 cells on a 24-well plate at a density of $2.0 \times 10^4$ cells·mL$^{-1}$ and cultivating them for a duration of 24 hours. The Transwell was then carefully inserted into the plate containing 0.5 mL of each sample, and it was cultured for an additional 24 hours. Finally, using a LIVE/DEAD staining kit, the cells were stained, and an Olympus IX83 fluorescence microscope was used to capture the fluorescence images. It was determined how well HA-TYR-R@P hydrogels could scavenge radicals using H$_2$O$_2$. More precisely, L929 cells with a density of $5.0 \times 10^4$ cells·mL$^{-1}$ were subjected to a culture containing $1 \times 10^{-3}$ m H$_2$O$_2$ for a duration of 12 h. A further 24 h were spent cultivating different samples after they were added. CCK8 kits were used to assess the cell viability in the end. RAW264.7 cells were inoculated with a 6-well tissue culture plate at a density of $1 \times 10^6$ wells and supplemented with 100 ng/mL lipopolysaccharides to incubate for 24 h to obtain polarized M1 macrophages. Polarize cells with HA-TYR, HA-TYR-P, HA-TYR-TYR-0.5%R@P and HA-TYR-1%R@P groups for 1 day. PBS was used to wash the above polarized cells, and M1 macrophages were specifically labeled with CD86 rabbit polyclonal antibodies and goat anti-immunoglobulin (IgG) labeled by Alexa Fluor 594 as secondary antibody; M2 macrophages were labeled with CD206 mouse polyclonal antibodies. FITC-labeled goat anti-mouse IgG as secondary antibody and DAPI were used to localize nuclear staining. Wash cells twice with PBS, fix 2.5% glutaraldehyde for 4 hours, and observe the conversion of macrophage phenotype M1 to M2 with a confocal laser.

2.8. Animals

The efficacy of hydrogel in promoting wound healing was validated in a model of full-layer skin wounds in rats infected with S. aureus. All animal procedures were conducted following the National Research Council’s Guide for the Care and Use of Laboratory Animals (8th edition, NIH Publication, 2011). SPF (Beijing) Biotechnology Co., Ltd. provided 60 female BALB/c mice, which were used in the wound repair tests. The experimental animals have access to enough pure water and food. All animal experiments were approved by the Experimental Animal Ethics Committee of the Chengdu University of Traditional Chinese Medicine. See the Supporting Information for detailed experimental procedures.

2.9. In Vivo Antibacterial and Wound Healing Experiments

After anesthesia, BALB/c mice (female, 18 ~ 21 g) received a 10 mm circular incision on the back. Subsequently, 100 µL of S. aureus ($10^6$ CFU/mL) was injected into the back wound of the mouse. HA-TYR-R@P hydrogel was applied to wounds that were infected. Mice were randomized into six groups (n = 10), and the wounds were treated in different ways: (1) Control group (S. aureus, physiological saline solution); (2) HA-TYR group (S. aureus, HA-TYR gel treatment); (3) HA-TYR-P group (S. aureus, HA-TYR-P gel treatment); (4) HA-TYR-R group (S. aureus, HA-TYR-R hydrogel treatment); (5) HA-TYR-0.5%R@P group (S. aureus, HA-TYR-0.5%R@P hydrogel treatment); (6) HA-TYR-1%R@P group (S. aureus, HA-TYR-1%R@P hydrogel treatment). Mice were monitored regularly for any signs of discomfort. Three days later, bacterial samples were collected from the infected wound using sterile cotton swabs. Cotton swabs were mixed with 1 mL of PBS, S. aureus was spread on nutrient agar, and the bacteria were allowed to grow for 24 hours at 37°C. High-definition digital camera photographs of the wound site were taken on days 0, 3,
6, 9, and 12. Image J software was used to measure wound area, and percentage wound healing was calculated. Finally, the mice were sacrificed and the corresponding skin samples and visceral tissue were taken for H&E, Masson and iNOS/CD206 immunohistochemical staining. Sixty BALB/c mice were used in the experiment.

2.10. Statistical Analysis

The experiment results were reported as the average ± standard deviation (SD) of a minimum of three separate experiments. Statistical analysis was performed using GraphPad Prism 7 software. The variation between groups was evaluated using an Unpaired two-tailed Student's t-test and one-way ANOVA. The statistical significance was expressed as P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) or P < 0.0001 (****).

3. Results and Discussion

3.1. Preparation and characterization of HA-TYR-R hydrogel

The HA-Tyr compound was synthesized by coupling the carboxyl groups of HA with the amino groups of tyramine using carbodiimide-mediated condensation. This conjugation was then used to develop an antibacterial and anti-inflammatory hydrogel. The 1H NMR analysis indicates that the HA-Tyr compound has a tyramine substitution degree of around 31.38% (Fig. 1a), which validate the successful conjugation. Subsequently, the phenol-rich polymers were crosslinked by HRP, forming the HA-Tyr-R hydrogels. Carbon-carbon or carbon-oxygen bonds are formed in this reaction when phenol groups are coupled at the ortho positions. The HA-Tyr-R hydrogels had a consistent, stable orange-red jelly-like appearance and well-stabilized physicochemical characteristics(Fig. 1b). Conversely, HA-TYR and HA-TYR-P are unable to form hydrogels when HRP and H2O2 are present because of the presence of NaHCO3 (pH 8.3, 0.2 M). Scanning electron microscopy reveals that HA-Tyr-R hydrogels generally possess a porous structure (SEM; Fig. 1c). We conducted a number of rheological tests to further ascertain the mechanical characteristics of HA-Tyr-R hydrogels. Hydrogel dressings, for instance, should be injectable and have good self-healing qualities. The study initially employed oscillatory shear rheology to investigate the reversibility of HA-Tyr-R hydrogels. For the purpose of analyzing the rheological properties of the HA-TYR-R@P hydrogel, changes in the storage modulus (G’i) and loss modulus (G”i) were observed under different testing conditions. After verifying the successful preparation of the hydrogel, it was observed that the storage modulus (G’) was higher than the loss modulus (G”) in the HA-TYR-R samples. The results depicted in Fig. 1d demonstrates that, when equal quantities of HRP and H2O2 are employed, the G’ of the HA-Tyr-R hydrogel is notably greater than that of the HA-Tyr gel. These findings indicate that rhein contributes to the development of the hydrogel structure, leading to a more compact network. In Fig. 1e, the dominant G’ over G” demonstrates that frequency scanning consistently shows that the HA-Tyr-R hydrogels remain in a gel state within the frequency range of 0.01–10 Hz. For the encapsulated PUE to remain in the wound infection region, the gel state must remain stable. The results of the step-strain test (Fig. 1f) showed that at a strain of 0.1%, the storage modulus (G’) was higher than the loss modulus
(G'), whereas at a strain of 300%, G' was lower than G". The material characteristics of HA-Tyr-R hydrogels were regained when the strain decreased from high to low, indicating the hydrogels' injectability and self-healing capabilities. We also investigated the viscoelastic properties of HA-Tyr-R hydrogels by conducting rotational rheology tests. The G' and G" variation with the frequency of HA-Tyr-R hydrogels was displayed, as Fig. 1e illustrates. G" was smaller than G' at both 25 and 37°C, and G' was roughly seven times larger on average. The hydrogel exhibited favorable shear thinning characteristics and was suitable for injection, as evidenced by the findings presented in Fig. 1f. Additionally, the data indicated a steady decrease in shear viscosity as the angular frequency increased. The hydrogels HA-Tyr-R demonstrated good thixotropy and self-healing properties, allowing them to be used as injection or external dressing hydrogels, as demonstrated by the rheological results above.

### 3.2. Antibacterial Activity of HA-TYR-R@P Hydrogels

Long-term nonclosure of chronic wounds exposes them to microbial infection[30]. Antimicrobial wound dressings not only look good, but they also serve as a basic barrier to keep out germs. Natural small molecules of plant origin are considered particularly effective in inhibiting bacteria[31]. Some studies have shown that berberine can be self-assembled with baicalin and wogonoside, respectively, into nanoparticles and nanobers, which showed different antibacterial effects[32]. RHE provides neuroprotection through anti-inflammation in the treatment of brain injuries[33, 34]. Despite this, RHE’s solubility is still low and it also shows low bioavailability due to glucuronidation metabolism in the liver, which hinders clinical transformation[35, 36]. Efforts have been made to develop polymeric microparticles and nanoparticles that contain RHE in order to enhance therapeutic effectiveness and minimize adverse effects. Drug loss during the fabrication process and early payload release result in reduced drug loading and undesirable systemic toxicity[21]. The enhanced RHE hydrogels possess both pH sensitivity and antibacterial properties, enabling controlled release and antibacterial effects. The antibacterial effect of the resulting hydrogel was investigated because RHE is one of the primary products in the antibacterial field. Antibacterial activity against both Gram-positive and Gram-negative microorganisms should be evident and broad-spectrum in the case of the HA-TYR-R@P. In order to validate this activity, we employed two commonly found microorganisms, namely staphylococcus aureus (S. aureus) and escherichia coli (E. coli), for antibacterial testing. Before incubating together for a duration of six hours, bacterial suspensions with a concentration of 106 CFU·mL − 1 were introduced into the preexisting hydrogel, which had been manufactured in 24-well plates. Figure 2a–b, e can demonstrate that even with a low concentration of HA-TYR-0.5%R@P hydrogel, the resultant hydrogels exhibited over 99% antibacterial efficacy against S. aureus. Considering Gram-positive bacteria, these findings imply that the hydrogel exhibited exceptional bactericidal activity. Regarding the Gram-negative bacterium E, the hydrogel additionally demonstrated strong bactericidal action. The HA-TYR-R, HA-TYR-0.5%R@P, and HA-TYR-1%R@P hydrogels showed 99.41%, 99.56%, and 99.71% antibacterial efficiency against E. Coli (Fig. 2c–d, e). When taken as a whole, these findings show that the HA-TYR-R@P hydrogel exhibits good broad-spectrum antibacterial activity in vitro at acceptable concentrations.
3.3. Biocompatibility and immune response properties of HA-TYR-R@P hydrogels

When HA-TYR-R@P hydrogel was used as a biomaterial, the main cause for concern was its biocompatibility. The biocompatibility and cellular responses of the HA-TYR-R@P hydrogel were assessed by a series of tests, such as the CCK8 cell viability assay, \( \text{H}_2\text{O}_2 \) scavenging assays, and LIVE/DEAD cell staining of L929 cells. There was no noticeable difference in cell viability among these groups after 24 hours, as Fig. 3a shows. The groups that underwent co-culturing for 48 hours showed excellent cell activities: HA-TYR-R, HA-TYR-0.5%R@P, and HA-TYR-1%R@P (Fig. 3b). The enhanced biocompatibility of hydrogel is a result of its ability to prevent the significant release of RHE and PUE due to molecular interaction. Furthermore, because free radicals are highly expressed in skin tissues, the skin and wounds with disrupted skin barriers are particularly vulnerable to oxidative damage. \( \text{H}_2\text{O}_2 \) was added as an oxidative stress model and was shown to reduce cell viability. However, the groups HA-TYR-R, HA-TYR-0.5%R@P, and HA-TYR-1%R@P were able to scavenge \( \text{H}_2\text{O}_2 \) and successfully fend off oxidative stress by utilizing the combined action of RHE and PUE (Fig. 3c). After performing LIVE/DEAD staining, it was observed that all samples exhibited no detectable cytotoxicity (Fig. 3e). The majority of cells displayed intense green fluorescence, indicating their viability, while only a small quantity of cells displayed weak red fluorescence, indicating cell death.

The subsequent healing of the skin is determined by the initial immune reaction to the dressing applied to the wound. Investigating the immune regulation of HA-TYR-R@P hydrogel was thus done using immunofluorescence staining analyses. The HA-TYR gel and HA-TYR-P gel effectively suppressed the transformation of macrophages into M1, as demonstrated by the immunofluorescence staining images of the macrophage mannose receptor CD206 (a marker for M2 macrophages, shown in green) and inducible nitric oxide synthase (a marker for M1 macrophages, shown in red) (Fig. 3e). The anti-inflammatory property of HA-TYR-R hydrogel was demonstrated by seeing a decrease in red fluorescence in HA-TYR gel and HA-TYR-P gel, as well as an increase in green fluorescence in HA-TYR-R hydrogel and HA-TYR-R@P hydrogel compared to the control group. The M1/M2 ratio was lower in the HA-TYR-1%R@P, HA-TYR-0.5%R@P, and HA-TYR-R groups due to the combined synergistic immunological effect of PUE and RHE. This indicates that the hydrogel has the capacity to modulate immunity (Fig. 3e). All things considered, HA-TYR-R@P hydrogel exhibited exceptional compatibility with living organisms and possessed the capability to regulate immune response by altering the ratio of M1/M2 macrophage polarization, inhibiting the expression of pro-inflammatory genes, and promoting the expression of anti-inflammatory genes. For the ensuing animal experiments, HA-TYR-1%R@P hydrogel was used in consideration of the viscoelastic and biological characteristics of these HA-TYR-0.5%R@P samples.

3.4. Wound Healing Ability of HA-TYR-R@P Hydrogel

To investigate the wound healing capabilities of the HA-TYR-R@P hydrogel and the relationship between infections and immunity in wound healing, a comprehensive model of infected wound healing was created by introducing S. aureus to the wound sites. The task was accomplished by utilizing six distinct
sets of samples. An analysis was conducted on six groups to demonstrate the synergistic impact of wound healing: HA-TYR, HA-TYR-P, HA-TYR-R hydrogel, HA-TYR-0.5%R@P hydrogel, and HA-TYR-1%R@P hydrogel. Control group (saline) was also included in the study. One 50 µL injection was given to each group right at the site of the wound. The wound healing rate was higher than that of the control, HA-TYR, HA-TYR-P, and HA-TYR-R hydrogel groups during the entire wound healing process because of the outstanding antibacterial property of the HA-TYR-R@P hydrogel group. Compared to the control group, the HA-TYR, HA-TYR-P, and HA-TYR-R hydrogel groups did not show as effective wound healing ability as the HA-TYR-R@P hydrogel group. The HA-TYR-R@P hydrogel group greatly reduced the size of the infected wound. It also managed the immune system, prevented excessive inflammation brought on by infections in skin wounds, and maintained an excellent antibacterial activity. In comparison to the other four groups, the HA-TYR-R@P hydrogel group demonstrated superior wound healing ability on days three and nine. After 9 days, the HA-TYR-1%R@P hydrogel group exhibited the biggest closed wound area, although it could be somewhat improved in the HA-TYR-P and HA-TYR-R hydrogel as well. Furthermore, in the continuous wound healing experiment, the HA-TYR-1%R@P hydrogel demonstrated the best wound healing ability (Fig. 4a, b). Images of the bacterial colony demonstrated the HA-TYR-R@P hydrogel's potent in vivo antibacterial activity. The HA-TYR and HA-TYR-P groups had high numbers of bacterial colonies, which suggested that the antimicrobial ability was undesired. Additionally, the HA-TYR-P group had more bacterial colonies than the control group, which may have been caused by a reduction in inflammation, which sped up bacterial growth (Fig. 4c). By using H&E staining, the histopathological structures of the mouse skins on day 12 were made visible (Fig. 4d). Over time, the capillaries constricted, the granulation tissue underwent fibrosis and turned into scar tissue, and the number of inflammatory cells (shown by the red arrow) steadily diminished throughout the final phases of skin healing. Day 12 wounds in control and HA-TYR groups still showed granulation tissue (black circle), which contained numerous inflammatory cells within the purple staining. Conversely, the treatment group receiving HA-TYR-R@P exhibited a notable decrease in inflammatory cells, and the presence of fibrotic scar tissue (shown by a blue arrow) was evident. Furthermore, in addition, the regeneration of mouse skin in the HA-TYR-R@P group was signaled by the appearance of hair follicles (green arrow), which resembled the typical 3D skin structure of mice. A significant number of regenerated hair follicles appeared in the HA-TYR-R@P group, whereas there was hardly any area with new hair follicles in control group, HA-TYR, HA-TYR-P, and HA-TYR-R groups. This indicated that the dermis was still healing. To increase tissue tensile strength and hasten healing during the remodeling phase, sufficient collagen deposition was essential. Consequently, to see newly formed collagen, Masson's trichrome staining (Fig. 4e) was ran. On day twelve, the wounds of the HA-TYR-R@P hydrogel treatment group showed a significant amount of collagen fibers, resulting in a somewhat regular epidermis and connective tissue. In contrast, the control and HA-TYR groups had only a little amount of deposited collagen fibers. The immunofluorescence staining of CD206 and iNOS demonstrated that the HA-TYR and control groups had low and high expression levels of CD206 and iNOS, respectively, indicating that infection may cause an overabundance of inflammation in the skin wound (Fig. 4f). A certain degree of inhibition of the strong inflammatory responses could be achieved by the infection of skin wounds and HA-TYR-P, HA-TYR-R, HA-TYR-R@P groups, as indicated by the expressions of iNOS and CD206 in the HA-TYR-P, HA-TYR-R, and HA-TYR-R@P
groups. The elevated bacterial growth rate observed in the HA-TYR-P group, as compared to the control group, may be attributed to the reduced expression of pro-inflammatory proteins and the enhanced expression of anti-inflammatory proteins. The remarkable anti-infection and immunological regulatory characteristics of the HA-TYR-R@P hydrogel were validated through the notably intense green fluorescence exhibited by CD206 and weak red fluorescence of iNOS, which further demonstrated the uninfected skin wound and relieved inflammatory response.

Accordingly, visceral histopathological sections assessed the biocompatibility of HA-TYR-R@P hydrogel in vivo. After administering HA-TYR-R@P hydrogel, the in vivo toxicity study revealed no pathological alterations in the kidney, liver, spleen, heart, or lung (Fig. 4g). Meanwhile, RHE and PUE have long been prescribed as therapeutic medications, and the data above demonstrated our confidence in the high biocompatibility of the HA-TYR-R@P hydrogel.

4. Conclusion

This study involved the design and creation of a hydrogel that was crosslinked using RHE, and it was used to encapsulate PUE. The calculated PUE values were included into a carrier hydrogel composed of tyramine-modified hyaluronic acid (HA-Tyr) mixed with RHE. This mixture was then combined with a catalytic system consisting of HPR/H$_2$O$_2$, which facilitated the interphenol coupling process to create a crosslinked HA-Try-RHE hydrogel that encapsulated the PUE. The HA-TYR-R@P hydrogel was created by including the naturally antibacterial component RHE, resulting in a synergistic effect that both fights bacteria and regulates the immune response. This hydrogel is intended for the treatment of infected skin wounds. The injectable and self-healing HA-TYR-R@P hydrogel has the ability to regulate immune response, promoting the healing of uninfected wounds. Additionally, it offers significant advantages in treating infected chronic wounds due to its excellent biocompatibility, antibacterial properties, and anti-inflammatory capacities. The HA-TYR-R@P hydrogel demonstrates promising characteristics as a dressing for treating infected wounds, with combined antibacterial and immune-regulating activities. This hydrogel also holds potential for use in chronic wound treatment in the future. The HA-TYR-R@P hydrogel not only showed bacteriostatic activity and appropriate wound dressing qualities, but also demonstrated excellent biocompatibility. The hydrogel was particularly advantageous due to the non-covalent link between RHE and HA-TYR, rather than relying on severe reaction conditions. This indicates that the fabrication technique was environmentally friendly, cost-effective, straightforward, and suitable for clinical applications. This work emphasizes the creation of a single-component, multipurpose hydrogel platform that eliminates the need for complex preparations and side effects associated with drugs. It also offers a universal method for the synthesis of RHE-based hydrogel for the treatment of wounds and raises awareness of Chinese herbal medicines in the development of biomaterials.

Declarations

Ethics approval and consent to participate
All animal experiments throughout the study were approved by the Experimental Animal Protection Association of Chengdu University of Traditional Chinese Medicine and all were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yu Zheng, Min Xu and Yongping Lu performed the data analysis; Yu Zheng and Zhibei Li performed the formal analysis; Xiaofang Li performed the validation; Yu Zheng wrote the manuscript. All authors read and approved the final manuscript.

References


**Scheme**

Scheme 1 is available in the Supplementary Files section.

**Figures**
Figure 1

Manufacturing of HA-Tyr-R hydrogels and their rheological characteristics. a) $^1$H NMR spectrum of HA-Tyr. b) The gel-sol transition of HA-Tyr-R hydrogels. c) SEM image of HA-Tyr-R hydrogels (Scale bars: 40 μm). d) Rheological time-sweep curves of HA-Tyr-0.5%R and HA-Tyr-1%R hydrogels at 25°C and 37°C. e) Rheological frequency-sweep curves of HA-Tyr-0.5%R and HA-Tyr-1%R hydrogels at 25°C and 37°C. f) The HA-Tyr-R hydrogels underwent three rounds of step-strain measurements, with low strain (0.1%) and high strain (300%) being applied. g) Composite viscosity variation at 25°C and 37°C with angular
frequency. The data were presented as mean ± SD, with n = 3. The unpaired t-test was used to analyze the values, with *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

Figure 2

The HA-TYR-R@P hydrogel demonstrates in vitro antibacterial against E. Coli and S. aureus at different doses. a) Statistical data of colonies, b) agar plates pictures, and c) Statistical data of colonies, d) agar plates pictures, and e) S. aureus and E. Coli stained with varying concentrations of hydrogel mats were shown in live-dead staining results. (Scale: 20 μm.) The data were presented as mean ± SD, with n = 3. The unpaired t-test was used to analyze the values, with *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.
Figure 3

Assessment of the biocompatibility and immune control of HA-TYR-R@P hydrogels in vitro. Viability of L929 cells cultured for 24 hours (a) and 48 hours (b) in HA-Tyr-R@P hydrogels. c) L929 cells’ viability after being cultured in HA-Tyr-R@P hydrogels with H$_2$O$_2$ treatment. d) LIVE/DEAD staining of L929 cells treated on the second day with HA-TYR hydrogel group, HA-TYR-P group, HA-TYR-R hydrogel group, HA-TYR-0.5%R@P group, and HA-TYR-1%R@P hydrogel group; red fluorescence indicates dead cells and green fluorescence indicate live cells. Bars of scale: 200 µm. e) iNOS (red), CD206 (green), and DAPI (blue) immunofluorescence staining of RAW264.7 cells following 48 hours of co-culturing with control, HA-TYR group, HA-TYR-P group, HA-TYR-R hydrogel group, and HA-TYR-0.5%R@P samples. Scale bars: 20 µm. The data were presented as mean ± SD, with n = 3. The unpaired t-test was used to analyze the values, with *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.
Figure 4

The ability of HA-TYR-R@P hydrogel to heal wounds. a) Images of the infected wound model at days 0, 3, 6, 9, and 12 showing the wound treated with saline (control), HA-TYR group, HA-TYR-P group, HA-TYR-R hydrogel group, HA-TYR-0.5%R@P hydrogel group, and HA-TYR-1%R@P hydrogel group. b) At days 0, 3, 6, 9, and 12, the wound closure rates of the control group, the HA-TYR group, the HA-TYR-P group, the HA-TYR-R hydrogel group, the HA-TYR-0.5%R@P hydrogel group, and the HA-TYR-1%R@P hydrogel group. c)
Images of the *S. aureus* colony taken on day three from the contaminated skin. On day 12, the following groups’ wound regeneration was assessed using H&E staining: control, HA-TYR group, HA-TYR-P group, HA-TYR-R hydrogel group, HA-TYR-0.5%R@P hydrogel group, and HA-TYR-1%R@P hydrogel group. e) Masson's trichrome staining at the site of the wound on day 12 of the healing process (scale bar, 50 μm). 
f) The regeneration tissues were stained with immunofluorescence. g) Histology images (scale bar, 200 μm) of the heart, liver, spleen, lung, and kidney of the *S. aureus*-infected mice following a 12-day treatment with HA-TYR-R@P hydrogel (healthy control). The data were presented as mean ± SD, with n = 5. The unpaired t-test was used to analyze the values, with *p < 0.05* and **p < 0.01**.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.png
- SupplementaryMaterial.docx