Chitosan Hydrogel Delivery System Containing Herbal Compound Functions as a Potential Antineuroinflammatory Agent

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ABSTRACT: Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is an anthraquinone compound mainly isolated from the herbal medicine rhubarb. It possesses a wide spectrum of pharmacological effects. However, the lack of sustained release properties and the poor bioavailability hinder clinical transformation. Hydrogel-based drug delivery system provides an ideal carrier to improve the release control and the therapeutic efficacy of drugs. Herein, we present a chitosan hydrogel for the delivery of rhein. This rhein–chitosan hydrogel (CS−Rh gel) exhibited superior characteristics including mechanical strength, sustained release, and low toxicity. For medical application, the enzyme-linked immunosorbent assay and Western blot analyses indicated that CS−Rh gel significantly suppressed the production of proinflammatory cytokines including TNF-α and IL-1β in lipopolysaccharide-induced BV2 cells. Additionally, CS−Rh gel blocked the neuroinflammation-related mitogen-activated protein kinase (JNK, ERK, and p38)-signaling pathways. Interestingly, these inhibitory effects at 48 h outperformed the pharmacologic actions at 24 h, showing that the CS−Rh gel exerted optimal sustained antineuroinflammation. This study highlights a novel chitosan hydrogel containing rhein used as a potential antineuroinflammatory agent.

INTRODUCTION

Drug delivery systems represent a promising therapeutic agent as carriers to enhance pharmaceutical efficacy. They commendably achieve long-term release of their payloads,1 realize drug retention in the injured tissues,2 avoid unacceptable toxicity,3 etc. These properties may replenish the therapeutic drawbacks, which the conventional administration of a drug through oral or intravenous has faced.4 Therefore, considerable attention has been focused on the applications of drug delivery systems in medical and biotechnological fields.5

With the development of drug delivery systems, the polymeric hydrogel has been provided as an attractive choice.6 Polymeric hydrogels are three-dimensional cross-linked polymers with a strong capacity for expansion following water adsorption. The polymeric hydrogels contain natural and synthetic polymers. In contrast, the natural polymers show better biocompatibility and biodegradability, as well as lower or null toxicity.7 Chitosan, a naturally derived amino polysaccharide obtained from the partial deacetylation of chitin, displays versatile characteristics such as biocompatibility, biodegradability, low toxicity, and antibacterial activity. Hence, the chitosan-based hydrogel is the ideal drug carrier for disease treatment.

Neuroinflammation is a prevalent pathological feature of neurological diseases, including traumatic brain injury, cerebral ischemia, and intracerebral hemorrhage.8−11 The release of proinflammatory factors triggers the death of neuronal cells, which is detrimental to the tissue repair of the brain. Despite the fact that several antineuroinflammatory agents have shown promising results, many of them failed in the clinical trials. Finding an antineuroinflammatory drug for the clinical application is urgently required and encouraged.

Fortunately, herbal medicines are now considered as potential bioactive candidates against diseases. The pharmacologist Youyou Tu, who won the 2015 Nobel Prize in Physiology or Medicine, has discovered the herbal medicine artemisinin for the treatment of malaria.12 Additionally, arsenic trioxide is recommended as the first-line treatment for acute promyelocytic leukemia.13 The incorporation of herbal medicine into the mainstream of medical systems has been commended by the World Health Organization. Hence, neuroscientists and pharmacists tend to explore natural products from the library of herbal medicines that function as antineuroinflammatory agents.14

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is a lipophilic anthraquinone compound mainly isolated from the herbal medicine rhubarb (Rheum palmatum L. or Rheum tanguticum Maxim, Dahuang in Chinese).15 Rhein performs pharmacological antineuroinflammation.16 Unfortunately, the
clinical translation has been hindered by various factors: (1) rhin has been demonstrated as a mechanism-based inhibitor of CYP450, which can completely inactivate drug-metabolizing enzymes, thus resulting in adverse effects; (2) moreover, rhin undergoes metabolism in the liver, in particular, the glucuronidation, exhibiting low bioavailability. Recently, Lai and Rogach highlight the hydrogels to enhance the delivery efficacy of herbal medicine. Thus, adopting chitosan-based hydrogel loads with rhin is expected to overcome the therapeutic challenges.

Herein, we fabricated a rhin–chitosan hydrogel (CS–Rh gel) and evaluated its mechanical strength with sustained release properties. For further medical study, lipopolysaccharide (LPS)-stimulated BV2 microglial cells were performed as an in vitro neuroinflammatory model. Finally, the antineuroinflammatory responses of the CS–Rh gel were tested.

**MATERIALS AND METHODS**

**Experimental Materials.** All chemical reagents and solvents were used as received without further purification unless otherwise noted. Chitosan (low molecular weight, 75–85% deacetylated) was purchased from Sigma-Aldrich. Rhin (purity > 98%, HPLC) was obtained from Natural Field Bio-Technique Co., Ltd. (Xi’an, China). All of the other reagents were of analytical grade. Deionized water (Milli-Q, 18 MΩ) was used throughout this study.

**Synthesis of CS–Rh Gel.** Chitosan solution was obtained by dissolving chitosan in 1% v/v acetic acid aqueous solution. The rhin powder was dissolved in 0.1 M NaHCO₃. All solutions were chilled in an ice bath for 15 min. Then, the rhin solution was added dropwise to the chitosan solution by evenly stirring at ice bath condition. The mixed solution was placed not less than 37 °C to allow the gelation. Gelation time was determined by the test tube inverted method. Here, the concentration of rhin in the hydrogel was controlled at 0–2.5 mg/mL. The concentration of chitosan was 6.67 mg/mL. Each final liquid solution was clear and homogeneous.

**Structure of CS-Rh Gel.** Micrographs of the external surface of CS–Rh gel were obtained by scanning electron microscopy (SEM, FEI HELIOS NanoLab 600i) and transmission electron microscopy (TEM, FEI Titan G2 60-300). The concentration of rhin in the solution was 1.4 mg/mL.

**Rheological Analysis.** Rheological characterization of the samples was carried out on a rotated rheometer (AR 2000ex, TA Instrument). We performed dynamic frequency sweep measurements (frequency: 0.1–100 Hz; strain: 0.1%; T = 25 °C), strain sweep measurements (strain: 0.01–100%; frequency: 10 rad/s; T = 25 °C), and time sweep measurements (the first step the strain was 0.1%, 120 s; the second step the strain was 40%; 20 s; frequency: 10 rad/s; circulation for 5 times). The concentration of rhin in the solution was 1.4 mg/mL.

**Fourier Transform Infrared (FTIR) Spectroscopy.** The FTIR spectra of the samples were recorded with a Perkin Elmer Spectrum One instrument. Potassium bromide was mixed with powdered samples to prepare thin films for studies. All spectra were recorded by transmittance mode at different wavelengths from 4000 to 400 cm⁻¹. Potassium bromide without samples was used as the control. The concentration of rhin in the solution was 1.4 mg/mL.

**Drug Release.** The solution was formed from phosphate-buffered saline (PBS) (pH = 7.4, 10 mmol/L) solutions containing rhin (1.1, 1.4, 1.7, and 2.0 mg/mL). A 3 mL sample of the release medium was soaked up from the sample vials periodically and replenished with a 2 mL fresh PBS to maintain a constant volume. The rhin in the medium was measured at 228 nm using the ultraviolet spectrophotometer (UV-2450, Shimadzu, Japan). A cuvette filled with fresh PBS was scanned as a blank to provide a spectral background in the ultraviolet determination.

**Cell Culture.** Mouse BV2 microglial cell lines were provided by the Institute of National Infrastructure of Cell Line Resource (Beijing, China). BV2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life) and 1% penicillin/streptomycin. The cells were maintained in a 5% CO₂-humidified incubator at 37 °C.

**Cytotoxicity Test.** The cells were seeded in the 96-well plates (1 × 10⁴/well cells) and allowed to attach overnight. A series of concentrations of CS–Rh gel (5, 10, and 20 μM) were added to each well for 24 h. Next, 10 μL of MTT (5 mg/mL in PBS, Sigma-Aldrich) was added to each well for an additional 4 h. After removing the supernatant, 150 μL of DMSO was added to each well to dissolve the resulting formazan crystals. The optical density was determined.

**LPS-Induced Inflammation and Drug Treatment.** BV2 cells were plated at a density of 1 × 10⁵/well in 6-well plates and incubated overnight to become near confluent. Cells were then incubated at 37 °C and stimulated with LPS (1 μg/mL) for 24 or 48 h in the presence or absence of CS–Rh gel. Experiments included cells grown in the medium alone as the control.

**Enzyme-Linked Immunosorbent Assay (ELISA).** The productions of TNF-α and IL-1β in the supernatant of the treated and untreated BV2 cell cultures were determined using ELISA kits (CUSABIO, Wuhan, China) according to the manufacturer’s instructions.

**Western Blot Analysis.** BV2 cells were washed with PBS and lysed with ice-cold RIPA buffer. After 30 min on ice, the lysates were centrifuged at 12 000 rpm for 15 min at 4 °C to produce whole-cell extracts. The supernatants were collected. The protein concentrations were measured using the bicinchoninic acid (Cwbiotech) method. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polycrylidan dine-difluoride membranes. The membranes were then blocked with 5% skim milk for 2 h before incubating with the following primary antibodies: rabbit anti-JNK (1:1000, Abcam), rabbit anti-p-JNK (1:200, Santa Cruz), rabbit anti-ERK (1:10 000, Abcam), rabbit anti-p-ERK (1:500, Abcam), rabbit anti-p38 (1:200, Proteintech), rabbit anti-p-p38 (1:200, Proteintech), rabbit anti-JNK (1:1000, Abcam), mouse anti-β-actin (1:4000, Proteintech), or rabbit anti-GAPDH (1:2000, Proteintech). After 1 h at room temperature. The immunobositive bands were detected using an enhanced chemiluminescent substrate (Thermo Fisher) and a Bio-Rad ChemiDoc XRS digital documentation system (Bio-Rad). The band density was quantified using Image J software. The amount of protein expression was presented relative to the levels of β-actin or ratio of phosphorylated protein: total protein.

**Immunofluorescence.** BV2 cells were fixed with 4% paraformaldehyde for 25 min and permeabilized with 0.3%
Triton X-100 for 30 min. Cells were blocked with 5% bovine serum albumin (BSA) for 1 h at 37 °C and followed by either of the primary antibodies: mouse anti-TNF-α (1:50, Proteintech) and rabbit anti-IL-1β (1:50, Proteintech) at 4 °C overnight. The cells were subsequently incubated with the corresponding Alexa Fluor 594-conjugated goat anti-mouse IgG (1:1000, Proteintech) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000, Proteintech) for 1 h at room temperature. The cells were counterstained with DAPI (Sigma-Aldrich) for 10 min at 37 °C. Images were obtained using a laser confocal microscope (TCS SP8 X & MP, Leica, Germany).

Statistical Analysis. All data in this study are presented as the mean ± standard deviation. Data were analyzed by a one-way analysis of variance. SPSS 18.0 was used for the statistical analyses. The criterion for statistical significance was $p < 0.05$.

RESULTS AND DISCUSSION

Morphology and Characteristics of the CS–Rh Gel. As shown in Figure 1A, the mixture of chitosan and rhein was liquid at 4 °C and formed a nonflowing gel at 37 °C. The gelation time was within 2 min. Temperature-induced gelation of chitosan/rhein solutions was observed. This thermosensitive property may help the CS–Rh gel undergo in situ gelation in

Figure 1. Morphology and rheological characteristics of the CS–Rh gel. (A) Images of the solution state at 4 °C and hydrogel state at 37 °C; (B) CS–Rh gel stayed its original gel state for up to 3 months; (C) SEM and TEM exhibited the polymer network morphology of the CS–Rh gel; (D–F) Oscillatory shear rheology of the CS–Rh gel. $G'$ is the storage modulus and $G''$ is the loss modulus.

Figure 2. FTIR spectra and drug release study. (A) FTIR spectra of chitosan and CS–Rh gel; (B) theoretical CS–Rh gel formation; and (C) CS–Rh gel exhibited sustained release property.
the body and easily fill irregular cavities. The representative photographs of the gel showed its saffron yellow and homogeneous appearance (Figure 1A). In addition, these hydrogels stayed their original gel state for up to 3 months under room temperature (Figure 1B). It indicates that CS−Rh gel is stable for over a long period. The interior morphology of the CS−Rh gel was examined in the freeze-dried state and observed by SEM and TEM (Figure 1C). The samples displayed highly connected polymer network morphology.

The frequency sweep of the hydrogel suggested that the values of the storage modulus \( G' \) were greater than the loss modulus \( G'' \). In addition, the \( G' \) and \( G'' \) values were freely dependent on frequency, confirming the solid-like behavior of the CS−Rh gel (Figure 1D). The results of strain-dependent oscillatory shear rheology showed a strain at a yield of 17.8% (this was the strain at the cross point of \( G' \) and \( G'' \), which described the transition of the gel network to a liquid state; Figure 1E). The step-strain measurements exhibited material properties of the CS−Rh gel recovered completely and rapidly when transitioning from a high magnitude strain (40%) to a low-magnitude strain (0.1%) for over five cycles of breaking and reforming (Figure 1F). This phenomenon reveals the dominant elastic nature of the gel. The good mechanical strength of the CS−Rh gel provides the possibility for its application in bioengineering.

**Structure of the CS−Rh Gel.** FTIR spectra of chitosan and CS−Rh gel were compared to explore the structure of CS−Rh gel (Figure 2A). As to the chitosan, the characteristic band of 3446 cm\(^{-1}\) was due to the existence of OH. It shifted to 3268.75 cm\(^{-1}\) and became broad after the CS−Rh gel formation. The apparent peaks at 1565.9 and 1415.4 cm\(^{-1}\) were attributed to the amido bond and CN stretching

![Figure 3](image-url) Cytotoxicity test and antineuroinflammatory responses of the CS−Rh gel in LPS-induced BV2 cells. (A) CS−Rh gel provided no significant effect on the viability of BV2 cells \( (N = 4/\text{group}); \) (B, C) ELISA exhibited that LPS dramatically increased the productions of TNF-α and IL-1β compared with the controls at both 24 and 48 h, while the high dose \((15 \mu\text{M})\) of CS−Rh gel reduced the levels of TNF-α by 43.08% and IL-1β by 45.72% at 24 h. When the treatment continued to 48 h, the hydrogel alleviated the expressions of TNF-α and IL-1β by 72.03 and 52.41%, respectively \( (N = 3/\text{group}); \) (D) representative Western blot expressions of TNF-α and IL-1β; (E, F) Western blot analysis showed that CS−Rh gel \((15 \mu\text{M})\) reduced the expressions of TNF-α by 57.43% and IL-1β by 39.44% at 24 h in the LPS-induced BV2 cells. When the treatment continued to 48 h, the hydrogel alleviated the levels of TNF-α and IL-1β by 74.96 and 41.81%, respectively \( (N = 3/\text{group}). \) *\( p < 0.05, \)**\( p < 0.01, \)***\( p < 0.001. \)

![Figure 4](image-url) Cellular immunofluorescence. Double immunofluorescence staining for expression and colocalization of TNF-α (red) and IL-1β (green) in LPS-induced BV2 cells in the presence or absence of CS−Rh gel (scale bar = 25 μm).
vibration, respectively. After gelation, the peaks shifted from 1565.9 to 1380.7 cm\(^{-1}\) and from 1415.4 to 1228.4 cm\(^{-1}\). The strengthened intensity of the peaks revealed the formation of hydrogen bonding between chitosan and rhein. FTIR analysis demonstrated that the amino group of chitosan has conjugated with the carboxyl group of rhein. In the presence of 1% acetic acid, CS–NH\(_2\) was converted into a protonated amino-saccharide unit (CS–NH\(_3^+\)) (Figure 2B,a). Additionally, rhein was completely dissolved in the NaHCO\(_3\) and ionized into an acid radical ion (Figure 2B,b). Subsequently, the interactions between the positive ion and negative ion took place, resulting in the gelation (Figure 2B,c). The experimental results suggest that it is feasible to constitute hydrogels through the interactions between chitosan and rhein.

**Drug Release Study.** The accumulated amount of rhein released from the hydrogel is presented in Figure 2C. The release profiles exhibited a fast release rate in the first 24 h and then experienced a moderate release within 100 h. The CS–Rh gel displayed a long-term sustained release property. As the concentration of rhein increased, the release rate declined. The reason may be that the carboxyl group derived from rhein has a high potentiality to increase the number of intermolecular hydrogen bonding in CS–Rh gel. This property tends to strengthen the cross-linked network, leading to the sustained release. Therefore, the release control of CS–Rh gel ensures the improved and prolonged pharmacological activities for medical application.\(^{22}\)

**CS–Rh Gel Provides No Affection on the Viability in Vitro.** The cytotoxicity of CS–Rh gel was assessed by the cell viability of BV2 cells. After MTT assay, the results indicated that there was no significant cytotoxicity on BV2 cells at the ranges from 0 to 20 \(\mu\)M (Figure 3A). According to the results of MTT assay, 3 and 15 \(\mu\)M of CS–Rh gel were selected for further medical studies. BV2 cell line, a well-known microglial cell line from the murine brain, exhibits both phenotypic and functional properties of reactive microglial cells.\(^{23}\) The present study illustrates that the CS–Rh gel shows high biocompatibility, implying that the hydrogel is an ideal potential delivery system for rhein.

**CS–Rh Gel Inhibits the LPS-Induced Inflammatory Responses in BV2 Cells.** To evaluate the effects of CS–Rh gel on proinflammatory cytokines in LPS-stimulated BV2 cells, we examined the expressions of TNF-\(\alpha\) and IL-1\(\beta\). The ELISA demonstrated that LPS dramatically increased the productions of TNF-\(\alpha\) and IL-1\(\beta\) compared with the controls at 24 and 48 h (Figure 3B,C). We further found that 15 \(\mu\)M of CS–Rh gel significantly suppressed the secretions of TNF-\(\alpha\) and IL-1\(\beta\) at both 24 and 48 h (Figure 3B,C). Interestingly, compared with the LPS group, the 15 \(\mu\)M of CS–Rh gel reduced the levels of TNF-\(\alpha\) by 43.08\% and IL-1\(\beta\) by 45.72\% at 24 h (Figure 3B,C). When the treatment continued to 48 h, the hydrogel alleviated the expressions of TNF-\(\alpha\) and IL-1\(\beta\) by 72.03\% and 52.41\%, respectively (Figure 3B,C). The observations suggest that these attenuations reinforced by CS–Rh gel at 48 h present a larger degree, which may reflect the sustained release of antineuroinflammatory responses. Hence, 15 \(\mu\)M of CS–Rh gel is chosen as the drug dose in the following experiments.

Further Western blot analysis confirmed that LPS notably increased the levels of TNF-\(\alpha\) and IL-1\(\beta\) compared with the controls at 24 and 48 h (Figure 3D–F). Compared with the LPS group, CS–Rh gel reduced the levels of TNF-\(\alpha\) by 57.43\% and IL-1\(\beta\) by 39.44\% at 24 h (Figure 3E,F). When the stimulation continued to 48 h, the hydrogel lowered the expressions of TNF-\(\alpha\) and IL-1\(\beta\) by 74.96 and 41.81\%, respectively (Figure 3E,F). It is evident that CS–Rh gel caused an excellent sustained released antineuroinflammation. Further cellular immunofluorescence labeled by TNF-\(\alpha\) (red) and IL-

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Figure 5. Effects of CS–Rh gel on the MAPK-signaling pathways in LPS-induced BV2 cells. (A) Representative Western blot expressions of p-JNK, JNK, p-ERK, ERK, p-p38, and p38; (B–D) Western blot analysis showed that the levels of the phosphorylations of JNK (at 24 and 48 h), ERK (at 48 h), and p38 (at 24 and 48 h) were notably increased in the LPS-stimulated BV2 cells. CS–Rh gel markedly suppressed p-JNK (at 48 h), p-ERK (at 48 h), and p-p38 (at 24 and 48 h) compared with the LPS group (\(N = 3/\text{group}\)). *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
β (green) confirmed the suppressive effects of CS−Rh gel on LPS-induced inflammation in BV2 cells (Figure 4).

Microglia, a type of resident macrophage cells in the central nervous system undergoes rapid activation in the process of neuroinflammation.24 In response to injury and other stimuli, the over-activated microglia cells migrate to the damaged areas, secreting huge amounts of proinflammatory mediators, such as TNF-α and IL-1β. These excessive inflammatory factors are detrimental to neurons, contributing to the pathogenesis of neuronal disorders.25 Previous studies have shown that regulating these proinflammatory mediators improves neuroinflammatory diseases.26 In this study, LPS-induced BV2 cell line was used as the in vitro model for the screening and evaluation of the antineuroinflammatory efficacy. We found that the antineuroinflammatory responses of the CS−Rh gel at 48 h were superior to the effects at 24 h. It illustrates that the sustained released property of the hydrogel contributes to the prolonged pharmacological activities.

**CS−Rh Gel Suppresses MAPK (JNK, ERK, and p38) Phosphorylation in LPS-Induced BV2 Cells.** We next investigated the effects of CS−Rh gel on MAPK-signaling pathways in LPS-induced BV2 cells (Figure 5A−D). LPS significantly increased the levels of the p-JNK (at 24 and 48 h), p-ERK (at 48 h), and p-p38 (at 24 and 48 h). CS−Rh gel markedly caused dephosphorylations of JNK (at 48 h), ERK (at 48 h), and p38 (at 24 and 48 h) compared with the LPS group (Figure 5B−D). The results suggest that CS−Rh gel obviously blocks the neuroinflammation-relevant MAPK-signaling pathways. Furthermore, the hydrogel triggers the controlled release inhibitions.

The MAPK-signaling pathways are considered as the classical pathways that regulate the neuroinflammatory responses.27 The activations of MAPKs lead to the massive releases of inflammatory factors (such as TNF-α and IL-1β) and initiate a strong oxidative stress response, consequently accelerating the inflammatory processes.28 The MAPKs mainly include JNK, ERK, and p38.29 Pharmacological interference with the MAPK-signaling pathways is considered to be a promising antineuroinflammatory strategy. In the present study, a drastic and rapid increase in the phosphorylation of MAPKs was found in LPS-stimulated BV2 cells. CS−Rh gel significantly deactivated MAPK (JNK, ERK, and p38) phosphorylations. The findings indicate that CS−Rh gel regulates the neuroinflammation-associated signaling pathways.

**CONCLUSIONS**

In summary, we developed a novel CS−Rh gel with superior characteristics including good mechanical strength, sustained release, and low toxicity. The hydrogel provides sustained antineuroinflammatory effects (Figure 6). This study highlights a novel chitosan hydrogel containing rhein from herbal medicine used as a potential antineuroinflammatory agent.

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**Author Contributions**

Y.W., J.Z., and J.Z. participated in the conception and design of the study. Y.Z., P.Z., J.Z., J.Z., and R.-R.S. carried out the experiments. T.T., J.-K.L., H.-J.C., R.-R.S., Y.W., J.Z., and J.Z. acquired and analyzed the data. J.Z. and J.Z. drafted the manuscript. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.
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