Directed self-assembly of herbal small molecules into sustained release hydrogels for treating neural inflammation

Jun Zheng1, Rong Fan2, Huiqiong Wu1,3, Honghui Yao4, Yujie Yan4, Jiamiao Liu4, Lu Ran5, Zhifang Sun1, Lunzhao Yi5, Li Dang6, Pingping Gan7, Piao Zheng8, Tilong Yang9, Yi Zhang1,2, Tao Tang2 & Yang Wang2

Self-assembling natural drug hydrogels formed without structural modification and able to act as carriers are of interest for biomedical applications. A lack of knowledge about natural drug gels limits their current application. Here, we report on rhein, a herbal natural product, which is directly self-assembled into hydrogels through noncovalent interactions. This hydrogel shows excellent stability, sustained release and reversible stimuli-responses. The hydrogel consists of a three-dimensional nanofiber network that prevents premature degradation. Moreover, it easily enters cells and binds to toll-like receptor 4. This enables rhein hydrogels to significantly dephosphorylate IκBα, inhibiting the nuclear translocation of p65 at the NFκB signalling pathway in lipopolysaccharide-induced BV2 microglia. Subsequently, rhein hydrogels alleviate neuroinflammation with a long-lasting effect and little cytotoxicity compared to the equivalent free-drug in vitro. This study highlights a direct self-assembly hydrogel from natural small molecule as a promising neuroinflammatory therapy.
Natural small molecules are regarded as promising drug resources due to their wide range of pharmacophores and high degrees of stereochemistry. Unfortunately, clinical treatments using these well-studied natural products are limited owing to the poor solubility and unsatisfying stability. For years, scientists focus on hydrogel-based drug delivery systems to improve the solubility and stability of natural small molecules. Numerous supramolecular hydrogels containing drug complexes have successfully been developed in the laboratory for inflammatory alleviation, wound repair, bacteria resistance, and tumor inhibition. However, clinical researchers continually find that the involvement of drug carriers may lead to poor biocompatibility and biodegradability, low loading efficacy, and potential side effects. Additionally, these biological materials from drug carriers require complex syntheses and are relatively expensive, which create great obstacles for effective clinical application.

To achieve optimal anti-inflammation through inhibition of the TLR4/NFκB signaling pathway, which essentially boosts the therapeutic efficacy and reduces the negative effects. These features trigger the rhein hydrogel to serve as a promising therapeutic agent for anti-neuroinflammation.

**Results**

**Morphology and gelation properties of rhein hydrogel.** In this study, the rhein direct self-assembly hydrogel was formed in PBS buffer by simple ultrasound with heating to obtain a homogeneous solution and subsequently cooled to room temperature. The minimum gelation concentration was 14.1 mM (Supplementary Fig. 1). The appearance of the hydrogel was a uniform orange-red. The scanning electron microscopy (SEM) images revealed that the scaffold was a 3D network composing of nanofibers. As can be observed in the transmission electron microscopy (TEM) and atomic force microscope (AFM) images, the nanofibers had an average diameter of approximately 30 nm with several micrometers in length (Fig. 1c and Supplementary Fig. 2).

Experimental tests indicated that pH intensively influenced the direct self-assembly process. The optimal pH values of the gel were between 8.0 and 9.4 (Supplementary Fig. 3a). Under this environment, we produced a translucent hydrogel. When the value of pH was above 9.4, the hydrogel began to collapse and became a blood red solution. The SEM images confirmed that the solution possessed a short, ribbon-like structure (Supplementary Fig. 3b). Within the ranges between pH 8.0 and pH 6.8, the sample was a viscous gel rather than a translucent gel. The microscopic morphology predominantly exhibited long fibers, while there were several short fibers distributed on the surface (Supplementary Fig. 3c). Once the pH values were set under 6.8, we observed precipitate formation. The SEM images displayed that these precipitates were the short, rod-like structures (Supplementary Fig. 3d). These results suggest that the formation of the hydrogel is highly dependent on the degree of carboxyl deprotonation of rhein molecules. To evaluate the role of the carboxyl group in gel formation, we sought to prepare the other anthraquinones from rhubarb, including emodin, chrysophanol, aloe-emodin, and physcion (Supplementary Fig. 4). We found that none of these anthraquinones formed hydrogels under the conditions tested, demonstrating that the carboxyl group plays an important role in the gelation process.

The rhein hydrogel showed multi-responsiveness to external environment changes (Fig. 1d). When the temperature increased to 40 °C, the hydrogel turned into solution. Once the temperature was adjusted to room temperature, the sol-to-gel phase transition occurred after several hours. In addition, the gel–sol transitions were triggered through vigorous shaking by hand, and the gel state was restored after standing within several minutes. Furthermore, the reversible properties of the rhein hydrogel were investigated by oscillatory shear rheology. When the strain exceeded 23.74%, the loss modulus (G″) was higher than the storage modulus (G′), revealing the transition from the gel to the solution state (solution state: G′ < G″, gel state: G′ > G″) (Fig. 1e). The step-strain test (Fig. 1f) revealed that G′ was higher than G″ at low strain 0.1%, and G′ was lower than G″ at higher strain 35%. The material properties of the rhein hydrogel recovered when transitioning from high strain to low strain, displaying the injectable and self-healing properties of the rhein hydrogel.

We also performed rotated rheology measurement to examine the viscoelastic properties. The dynamic frequency sweep showed that the values of G′ were 5 times larger than those of G″ (Fig. 1g). Moreover, there was a weak dependency with frequency. Additionally, the G′ was much greater than the G″ during the entire process, according to the dynamic time sweep data (Fig. 1h). The excellent thixotropy and thermo-reversibility indicate that the rhein hydrogel can serve as an injectable hydrogel.
Fig. 1 Molecular formula of rhein and characterization of the rhein hydrogel. a Chemical structures of rhein and digital images of the rhein hydrogel. b SEM image of the rhein hydrogel, scale bar: 2 μm. c TEM image of the rhein hydrogel, scale bar: 500 nm. The upper right is a partial enlarged view, scale bar: 20 nm. d Reversible gel-sol transitions of rhein hydrogel triggered by shear stress and temperature. e Strain-dependent oscillatory shear rheology of the rhein hydrogel with a fixed frequency of 10 rad s⁻¹. f Step-strain measurements of the hydrogel over five cycles at low strain (0.1%) and high strain (35%), frequency 10 rad s⁻¹. g Dynamic frequency sweep of the rhein hydrogel, measured at 0.1% strain. h Dynamic time sweep of the rhein hydrogel, at the strain of 0.1% and at the frequency of 10 rad s⁻¹. The concentration of hydrogel was 5 mg mL⁻¹ (17.6 mM), T = 25 °C.
The directly self-assembly mechanism of rhein hydrogel. We used the mass spectrometry (MS) system to explore the structure of rhein aggregates, which is a powerful tool to investigate the assembly of aggregates. The monomers (m/z 283.0249), dimers (m/z 589.0388), trimers (m/z 895.0514), tetramers (m/z 1201.0647) and higher-order aggregates were observed from MS (Fig. 2a, b). These aggregates existed as sodium salt clusters, the dimers were the predominant aggregate ions, indicating that the dimer is relatively stable relative to other aggregates. These results were further proved by the MS/MS analysis. We found the fragment ions of the dimer, rhein sodium salt (m/z 305.0061) and rhein monomer from the MS/MS spectrum of higher-order aggregates (Fig. 2c-g). These results demonstrate that rhein monomers and sodium salt of rhein are the building block of rhein nanostructures.

To shed light on the self-assembly process of rhein fibrils, we implemented spectroscopy studies. The FT-IR results suggested that there was a typical hydrogen bond absorption peak of O-H at 3436 cm⁻¹. During the gelation, the peak of O-H at 3436 cm⁻¹ shifted to 3429 cm⁻¹, accompanied by an enhanced intensity in the hydrogel phase. On the other hand, the peak of C=O at 1704 cm⁻¹ shifted to 1662 cm⁻¹ (Supplementary Fig. 5). These shifts indicate that hydrogen bonds are involved in the self-assembly process.

The concentration-dependent UV/Vis absorption study displayed the characteristic anthraquinone group peak, which underwent a redshift from 418 to 503 nm as the concentration of rhein increased (Fig. 3a), which was assigned to the π-π interactions of the anthraquinone structure. This phenomenon suggests that the aggregate configuration changed from H-type aggregation to J-type aggregation.

We next performed fluorescence spectroscopy analysis to assess whether π-π stacking played a role during the self-assembly process. As shown in Fig. 3b, an emission peak centered at 601 nm appeared in the solution, while the center of the emission peaks from the rhein hydrogel at 601 shifted to 616 nm. As the concentration of the hydrogel increased, the fluorescence intensity decreased and the peak red shifted (Supplementary Fig. 6). The fluorescence tended to quench due to the aggregation of rhein molecules. The peak redshift was attributed to the presence of the anthraquinone excimt which mainly contributed to the self-assembly process through π-π interactions. X-ray diffraction (XRD) also confirmed this result. The apparent peak at d = 3.4 Å was a typical distance of π-π stacking interactions between the two molecules.

We further employed circular dichroism (CD) spectra to explore the molecular packing of rhein within the hydrogel. The CD spectra exhibited strong negative peaks at 228 nm and 412 nm (Fig. 3d). These observations suggest that the chiral packing of rhein within the hydrogel is formed in the self-assembly process. The negative peaks revealed the left-hand helical arrangement. With the concentration of rhein hydrogel increased, the CD spectra displayed a strong negative bisignate CD signal between 210 and 300 nm. These CD signal peaks showed an obvious redshift. The shift phenomena show that the J-aggregation of rhein occurs in the rhein hydrogel.

Combining experimental results with theoretical calculation, we proposed a possible self-assembly process of rhein hydrogel. The gelation of rhein was highly dependent on pH. When the pH value was set between 8.0 and 9.4, the rhein hydrogel was obtained. Under this condition, certain molecules were deprotonated to form rhein sodium salt. Rhein monomer and rhein sodium salt were together assembled to form dimer and higher-order aggregates. The two molecules were arranged in a J-type aggregation manner by π-π stacking and hydrogel bonding to form a dimer. Due to the electrostatic repulsion between the carboxylic acid ions, two molecules tended to be arranged in opposite direction. The density functional theory (DFT) calculations also supported this conformation (Supplementary Fig. 7). Subsequently, dimers were further assembled into trimers, tetramers and higher-order aggregates. The rhein molecules continually added to the per-existing aggregates in a left helix fashion, resulting in the formation of the nanofiber with a left-handed helical configuration. The nanofibers further crosslinked to form 3D networks (Fig. 3e).

Assessments of sustained release and cytotoxicity. We examined the sustained release of rhein hydrogel within a series of concentrations. Figure 4a confirmed the release profile. There was a fast release during the first 12 h, and then the release of rhein showed a gradual process. Regardless of high concentration or low concentration, the release rate was up to 70% after 24 h, which was attributed the good solubility of the hydrogel and the brittleness of the fibers. As the gel concentration increased, the release rate decreased, accompanied by the extension of the release time. Moreover, the cumulative release percentage at low concentrations reached 90% within 36 h. Only a small amount of rhein was released from 36 to 72 h. The cumulative release rate under high concentrations merely came to 80% within 36 h and the release rate reached 88% after sustained released to 72 h. The higher concentration of rhein hydrogel which possessed a closer fibers network, resulted in more durable release of the rhein molecules from hydrogel. These properties facilitate the rhein hydrogel to serve as a potential biomedical application.

Stability is a key indicator to evaluate whether a drug can potentially be used in clinical application. The rhein hydrogel maintained a good stability after being stored at room temperature for 3 months (Supplementary Fig. 8). In order to investigate the stability of the rhein hydrogel in cells, we used liquid chromatography-mass spectrometry (LC-MS) to detect the concentration of rhein in the BV2 cells treated with rhein hydrogel and free-drug for 3 h, 12 h, 24 h, 48 h, and 72 h, respectively. As shown in Fig. 4b, the concentration of rhein from hydrogel group was higher than the free-drug group at each time point. Furthermore, the dimers were merely observed in rhein hydrogel group (Supplementary Fig. 9). These data suggest that rhein nanofiber is more likely to penetrate into cells than free-drug form. In addition, we found that the concentration of free-drug reached the top at 24 h and then decreased at 48 h. While there was a plateau during 24–48 h in rhein hydrogel group, indicating that the rhein hydrogel is more stable in the cells.

To evaluate the toxicity, we executed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by assessing the effect of the drug concentration on cell viability. Figure 4c suggested that the rhein hydrogel and the equivalent free-drug reached the top at 24 h and then decreased at 48 h. We then continued to 48 h (Fig. 4d), 40 µM of the free-drug significantly diminished the BV2 cells viability by 22% compared with the rhein hydrogel. Interestingly, the rhein hydrogel at various concentrations did not affect the viability of BV2 cells. The results demonstrate that the cytotoxicity of the rhein hydrogel is lower than that of the free-drug during the slow and sustained release.

Rhein hydrogel exerts better anti-neuroinflammation. For medical transformation, we used a LPS-induced BV2 cell line as an in vitro model to estimate the anti-neuroinflammatory effects of the rhein hydrogel. During neuroinflammation, brain microglia underwent morphological changes and proliferation, triggering the excess production of various proinflammatory factors. We therefore conducted an MTT assay to evaluate the effects of the rhein hydrogel on BV2 cells. The results demonstrated that the rhein hydrogel significantly reduced the release of proinflammatory factors such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). The anti-inflammatory effects of the rhein hydrogel were confirmed by the decreased production of these cytokines.

In summary, we have developed a rhein hydrogel with sustained release properties and anti-neuroinflammatory effects. The rhein hydrogel exerts better anti-neuroinflammation by reducing the release of proinflammatory factors and maintaining cell viability. The rhein hydrogel could be a promising candidate for the treatment of neuroinflammatory diseases.
mediators. TNF-α and IL-1β have been recognized as the key factors in neuroinflammation. Microglia cells are primed by neuroinflammatory disorders to produce exaggerated responses to IL-1β and TNF-α. Thus, we primarily focused on the drug effects on these two cytokines. We tested the rhein hydrogel in a therapeutic setting in LPS-stimulated BV2 cells. LPS led to the marked accumulation of TNF-α and IL-1β compared to the control (Fig. 5). After 24 h treatment, the hydrogels and the

Fig. 2 Mass spectrometric analysis of the rhein hydrogel. a, b MS analyses of the rhein hydrogel. c MS/MS analyses of monomer [M−H]−. d MS/MS analyses of dimer [2M−2H+Na]−. e MS/MS analyses of trimer [3M−3H+2Na]−. f MS/MS analyses of tetramer [4M−4H+3Na]−. g MS/MS analyses of pentamer [5M−5H+4Na]−.
equivalent free-drug dosing resulted in the obvious elimination of TNF-α and IL-1β, as confirmed by the hypointensity of red (TNF-α) and green (IL-1β) fluorescent protein-positive cells co-expressed in the microglia cytoplasm (Fig. 5a). Similarly, subsequent western blotting and enzyme linked immunosorbent assay (ELISA) revealed that the levels of TNF-α and IL-1β were dramatically reduced with both drugs compared to the LPS-treated group (Fig. 5b–f). In addition, the results of ELISA assays showed that both drugs notably decreased the releases of IL-6, IL-12, and iNOS compared to the LPS-treated group (Fig. 5g–i).

To observe the sustained release efficacy, the treatment continued for 48 h. We found that the hydrogel significantly lowered the levels of TNF-α, IL-6, IL-12, and iNOS compared with the equivalent free-drug dosing. (Fig. 5). The observations reveal that the rhein hydrogel exhibits better neuroinflammatory prevention than the free-drug through the direct self-assembly nanofibers under sustained release control.

**Rhein hydrogel enhances TLR4/NFκB inhibition.** To further elucidate the anti-inflammatory pathways of the rhein hydrogel, it is critical to explore the underlying molecular mechanisms. Neuroinflammation is mainly implicated in the activation of p38, PI3K/Akt and TLR4/NFκB signaling pathways. We used western blotting analyses to investigate the anti-inflammatory mechanisms of rhein hydrogel and the free drug. We studied the effects of rhein and rhein hydrogel on TLR4/NFκB signaling pathways. Following activation of BV2 cells by LPS, increasing TLR4 expression followed by the enhancement of phosphorylation and degradation of IκBα in the cytoplasmic extracts were observed compared to the control (Fig. 6b–e). NFκB activity was evaluated according to the nuclear translocation of the p65 subunit of NFκB. The findings revealed that LPS induced phosphorylation of IκBα, further resulting in the remarkable shift of NFκB p65 into the nucleus. The presence of rhein hydrogel significantly prevented increases in TLR4, phosphorylation of IκBα and nuclear translocation of p65. In particular, during the phase of sustained release (48 h), the rhein self-assembly hydrogels better inhibited the TLR4/NFκB pathway than did the equivalent free-drug dosing (Fig. 6b–e). We also surveyed the effects of the rhein hydrogels and free-drug on the other neuroinflammatory signaling pathways, including p38 and PI3K/Akt (Supplementary Figs. 10 and 11). The results indicate that no
better inhibitions of p38 and PI3K/Akt phosphorylations induced by the rhein hydrogel were found compared to the free drug at 48 h. Our work demonstrates that the better therapeutic effects induced by the rhein hydrogel under sustained release are mainly associated with TLR4/NF-κB inhibition.

**Discussion**

In this work, we have presented rhein-based direct self-assembly hydrogel. The rhein hydrogel exhibits excellent solubility, sustained release properties, low toxicity, and reversible stimulus-responsive properties to shear stress and temperature. As a directed self-assembly drug hydrogel, the rhein hydrogels significantly enhance anti-neuroinflammation compared with the free-drug through downregulation of proinflammatory cytokines and neurotoxic factors. The superior sustained release anti-neuroinflammatory effects from the rhein self-assembly hydrogels are associated with the inhibition of the TLR4/NFκB signaling pathway. The directed self-assembly hydrogels display superior solubility, optimal therapeutic efficacy, and fewer adverse effects. These properties facilitate the rhein hydrogel to serve as a potential anti-neuroinflammatory agent.

The gelation pathway is of key importance to append this unique gel system to other natural small molecules with low solubility. Our experiments indicate that the rhein hydrogel was formed by the self-assembly of rhein molecules through π-π stacking, hydrogen bonds, and other non-covalent interactions. Based on the experimental results, a possible self-assembly mechanism of the rhein hydrogel was proposed. The evidence demonstrates that the rhein monomer is capable of accomplishing self-assembly processes without any structural modification or delivery carrier. This provides a potential guide for the self-assembly of other natural small molecules.

Understanding the anti-neuroinflammatory effects of the rhein hydrogel is the core of this study. It is notable that with treatment of free-drug and hydrogels for 24 h, both drugs exhibit similar anti-neuroinflammatory effects without obvious cytotoxicity by reducing proinflammatory mediators, including TNF-α, IL-6, IL-12, and iNOS. More interestingly, as the treatment continues for 48 h, the rhein diminishes microglial cell viability at 40 μM, whereas the rhein hydrogel does not affect cell viability. Furthermore, the rhein hydrogel markedly lowers the levels of TNF-α, IL-6, IL-12, and iNOS compared with the rhein monomer. The results reveal that the rhein hydrogel provides less cytotoxicity and better neuroinflammatory prevention than the free-drug under the sustained release control.

It is critical to explore how the rhein hydrogel interacts with protein targets to achieve the better therapeutic effects. Our work illustrates that both the rhein hydrogel and the free-drug block p38, PI3K/Akt, and TLR4/NFκB pathways to alleviate neuroinflammatory responses. Particularly, the better therapeutic effects induced by the rhein hydrogel under the sustained release are primarily associated with TLR4/NFκB inhibition, rather than p38 and PI3K/Akt pathways. This distinction may be attributed to the nanofiber structure of the rhein hydrogel. Moreover, we further use molecular docking techniques to elucidate the underlying mechanism. We confirm that TLR4 is a potential druggable target of rhein (Fig. 6a). This specific recognition facilitates the transfer of LPS to MD-2 protein, further activating the downstream components, including NFκB, and leading to the excess releases.

**Fig. 4** Rhein hydrogel performed better sustainable release and lower cytotoxicity. a In vitro release profiles of rhein hydrogel, the concentrations of rhein hydrogel were 4 mg mL$^{-1}$ (14.1 mM) and 6 mg mL$^{-1}$ (21.1 mM), respectively. The gels were incubated with PBS buffer solution (pH 7.4) at 37 °C. b The curve of the concentration of rhein in the cells over time. c, d Cell viability (24 h and 48 h) of BV2 cells incubated with different concentrations of rhein hydrogel and rhein, the concentrations of the samples range from 0 to 40 μM. Values are expressed as the means ± SEM (n = 3). One-way ANOVA with Tukey’s post hoc test. *p < 0.05. Error bars represent SEM.

| Concentrations (μM) | Time (h) | Cell viability (%) | Rhein | Rhein gel |
|---------------------|----------|-------------------|-------|-----------|
| 0                   |          | 0                 |       |           |
| 5                   |          | 20                |       |           |
| 10                  |          | 40                |       |           |
| 20                  |          | 60                |       |           |
| 40                  |          | 80                |       |           |

| Concentrations (μM) | Time (h) | Cumulative release (%) | Rhein | Rhein gel |
|---------------------|----------|------------------------|-------|-----------|
| 0                   |          | 0                      |       |           |
| 12                  |          | 20                     |       |           |
| 24                  |          | 40                     |       |           |
| 36                  |          | 60                     |       |           |
| 48                  |          | 80                     |       |           |
| 60                  |          | 100                    |       |           |

| Concentrations (ng.mL$^{-1}$) | Time (h) | Cell viability (%) | Rhein | Rhein gel |
|------------------------------|----------|-------------------|-------|-----------|
| 0                             |          | 100               |       |           |
| 5                             |          | 90                |       |           |
| 10                            |          | 80                |       |           |
| 20                            |          | 70                |       |           |
| 40                            |          | 60                |       |           |

The gelation pathway is of key importance to append this unique gel system to other natural small molecules with low solubility. Our experiments indicate that the rhein hydrogel was formed by the self-assembly of rhein molecules through π-π stacking, hydrogen bonds, and other non-covalent interactions. Based on the experimental results, a possible self-assembly mechanism of the rhein hydrogel was proposed. The evidence demonstrates that the rhein monomer is capable of accomplishing self-assembly processes without any structural modification or delivery carrier. This provides a potential guide for the self-assembly of other natural small molecules.

Understanding the anti-neuroinflammatory effects of the rhein hydrogel is the core of this study. It is notable that with treatment of free-drug and hydrogels for 24 h, both drugs exhibit similar anti-neuroinflammatory effects without obvious cytotoxicity by reducing proinflammatory mediators, including TNF-α, IL-6, IL-12, and iNOS. More interestingly, as the treatment continues for 48 h, the rhein diminishes microglial cell viability at 40 μM, whereas the rhein hydrogel does not affect cell viability. Furthermore, the rhein hydrogel markedly lowers the levels of TNF-α, IL-6, IL-12, and iNOS compared with the rhein monomer. The results reveal that the rhein hydrogel provides less cytotoxicity and better neuroinflammatory prevention than the free-drug under the sustained release control.

It is critical to explore how the rhein hydrogel interacts with protein targets to achieve the better therapeutic effects. Our work illustrates that both the rhein hydrogel and the free-drug block p38, PI3K/Akt, and TLR4/NFκB pathways to alleviate neuroinflammatory responses. Particularly, the better therapeutic effects induced by the rhein hydrogel under the sustained release are primarily associated with TLR4/NFκB inhibition, rather than p38 and PI3K/Akt pathways. This distinction may be attributed to the nanofiber structure of the rhein hydrogel. Moreover, we further use molecular docking techniques to elucidate the underlying mechanism. We confirm that TLR4 is a potential druggable target of rhein (Fig. 6a). This specific recognition facilitates the transfer of LPS to MD-2 protein, further activating the downstream components, including NFκB, and leading to the excess releases.
**Fig. 5** Rhein and rhein hydrogel alleviated neuroinflammation in LPS-induced BV2 cells. 

- **a** Confocal microscopy images from BV2 cells (treated with control, LPS, rhein and rhein hydrogel). Immunofluorescence staining is shown. Cell nuclei are shown in blue (DAPI), TNF-α is shown in red, IL-1β is shown in green, and yellow labeling represents co-localization. Scale bar, 15 μm.

- **b** Representative western blots of TNF-α and IL-1β proteins at 24 h and 48 h. The uncropped and unprocessed scans of blots are presented in Supplementary Fig. 12.

- **c** Quantiﬁcations of TNF-α at 24 h and 48 h. 

- **d** Quantiﬁcations of IL-1β proteins at 24 h and 48 h.

- **e** ELISA assays for determinations of TNF-α (e), IL-1β (f), IL-6 (g), IL-12 (h) and iNOS (i) levels at 24 h and 48 h. Values are expressed as means ± SEM (n = 3). One-way ANOVA with Tukey’s post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM.
of proinflammatory cytokines and neurotoxic factors.\(^{49-51}\) Therefore, the TLR4/MD-2/LPS complex represents a symmetrical manner to stimulate the proinflammatory responses. Inhibiting TLR4 dimerization is considered a key maneuver to regulate neuroinflammatory disorders.\(^{52,53}\) This work reveals that strong hydrogen bonding and \(\pi-\pi\) interactions exist between TLR4 and the residues of TLR4, including Ser120, Phe121, Gly123, Ile124, Lys125, and Lys126 (Fig. 6a). The rhein binds to MD-2 (subunit of TLR4 receptor) and forms a steric hindrance effect, which results in the inability of TLR4 to efficiently identify LPS, thereby inhibiting the NF\(\kappa\)B signaling pathway. Compared to the free drug, rhein hydrogel consists of nanofibers with relatively larger size and orderly structure, which has a strong tendency to prolong the circulation time and allow for increased accumulation.\(^{54,55}\) On the other hand, the nanofibers are more easily uptaken by cells than the free-drug form, providing more opportunity for these molecules to bind to TLR4. Furthermore, more hydrogen bonds and \(\pi-\pi\) interactions from the aggregates contribute to the tight binding of TLR4. Hence, this hydrogel remarkably obscures the active site of TLR4 and blocks the access of the substrate, leading to better long-lasting effects and lower toxicity.\(^{56}\) This illustration is in accord with the results of western blot in this study. Once LPS binds to TLR4 on the surface of microglia, it causes the translocation of p65 into the nucleus and binds to the DNA binding site. This regulates the transcription of its target genes, triggering the expression of pro-inflammatory enzymes and cytokines. The inhibition of TLR4 by the rhein nanofiber deactivates the downstream NF\(\kappa\)B signaling pathway, leading to the downregulation of inflammatory mediators and cytokines. The present study shows that the superior inhibition of TLR4/NF\(\kappa\)B at 48 h may result from the slow dissolution and continuous release of the rhein molecules from the nanofibers. Accordingly, the rhein hydrogel can be used as a neuroinflammation-targeting agent.

In summary, we have explored direct self-assembly hydrogels of a natural product without any delivery carriers or structural modifications through hydrogen bonds, \(\pi-\pi\) interactions and electrostatic interactions. The rhein hydrogel exhibits excellent stability, sustained release properties and reversible stimuli-responsive properties. The hydrogel consists of a 3D nanofiber network structure that prevents premature degradation. Moreover, it easily enters cells, leading to an intensive binding to the active site of toll-like receptor 4. These advanced properties enable the rhein hydrogel to significantly dephosphorylate I\(\kappa\)B\(\alpha\), inhibiting the nuclear translocation from the p65 at NF\(\kappa\)B signaling pathway in LPS-induced BV2 microglia. In particular, the rhein hydrogel markedly alleviates neuroinflammation with a long-lasting effect without cytotoxicity compared with the equivalent free-drug dosing in vitro. To the best of our knowledge, this work provides a paradigm for discovering direct self-assembly hydrogels formed by natural small molecules to significantly promote therapeutic effects with no obvious cytotoxicity (Fig. 7).

**Methods**

**Materials.** Rhein was obtained by Xi’an Natural Field Bio-Technique Co., Ltd. Sodium bicarbonate NaHCO\(_3\) was provided from Sinopharm Chemical Reagent Co., Ltd. Phosphate buffer solutions (PBS) were purchased from Xiya Reagent (Chengdu, China). MTT reagent, dimethylsulfoxide (DMSO), destination access point identifier (DAP), polyacrylamide and sodium dodecyl sulfate were purchased from Sigma. Ultrapure water (\(>18\,\text{MO}\)) was used. All the reagents were used without further purification throughout this study.

**Preparation of the rhein hydrogel.** Rhein was dissolved in NaHCO\(_3\) (pH 8.3, 0.2 M) or PBS (pH 8.0–9.4, 0.1 M) followed by ultrasound or heat to obtain a homogeneous solution. Subsequently, the solution was cooled to room temperature

**Fig. 6** Rhein and rhein hydrogel inhibited TLR4/NF\(\kappa\)B pathway in LPS-induced BV2 cells. a Molecular docking analysis of the interaction between rhein and TLR4. Molecular docking model illustrated the interaction between rhein and the active sites of TLR4, showing the existences of intermolecular hydrogen bonds and \(\pi-\pi\) interactions. b Rhein and its hydrogel blocked the TLR4/NF\(\kappa\)B pathway, as shown by western blot analysis. Representative western blots of TLR4 in total proteins, I\(\kappa\)B\(\alpha\) and p-I\(\kappa\)B\(\alpha\) in cytosolic extracts, and p65 in nuclear extracts after treatment with rhein or rhein hydrogel for 24 and 48 h. The uncropped and unprocessed scans of blots are presented in Supplementary Fig. 13. c-e Quantification of TLR4 (c), p-I\(\kappa\)B\(\alpha\)/I\(\kappa\)B\(\alpha\) (d) and p65 (e) according to densitometric analysis. The results are presented as a ratio of TLR4/GAPDH, p-I\(\kappa\)B\(\alpha\)/I\(\kappa\)B\(\alpha\) and p65/Lamin B. Values are expressed as the means \(\pm\) SEM (n = 3). One-way ANOVA with Tukey’s post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars represent SEM.
and accompanied by ultrasound. A uniform stable hydrogel was obtained after 5 min, and the critical gel concentration was 4 mg mL\(^{-1}\) (14.1 mM).

**Rheology test.** The rheological studies were carried out on a rotated rheometer (AR 2000ex, TA Instrument, USA). The dynamic frequency sweep was measured at 0.1% strain, and the frequency was between 0.01 and 100 Hz. The dynamic time sweep was measured at 0.1% strain and the time was kept at 2400 s. The strain-dependent oscillation was measured at 0.1–100% strain. The step-strain rheological experiment was performed in two steps. First, the rhein hydrogel was tested with a strain of 0.1% (step 1). Then, the strain was changed from 0.1 to 35% and remained at 35% for 1 min to completely damage the gel (step 2). Further, the strain was reduced from 35 to 0.1% again and remained at 0.1% for 3 min to observe whether the gel was restored. During the entire test, the frequency remained at 10 rad s\(^{-1}\) and the temperature was kept at 25 °C. All measurements were performed at 25 °C and at a concentration of 5 mg mL\(^{-1}\) (17.6 mM) hydrogel.

**Analysis of rhein aggregates by mass spectrometry.** We studied the structure of the self-assembled hydrogel by ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC-Q-Orbitrap/MS/MS). The UHPLC-Q-Orbitrap-MS system consisted of a Dionex U3000 UHPLC system (Thermo Fisher Scientific, MA, USA) and a high-resolution Q-exactive focus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The Dionex U3000 UHPLC system was equipped with a quaternary Series RS pump, a degasser, an auto sampler, and a column compartment. The chromatographic system was coupled to the mass spectrometer with a heated electrospray ionization source (HESI). Xcalibur 3.0 (Thermo Fisher Scientific, Bremen, Germany) was used for UHPLC-MS control and data handling.

Chromatographic separation was performed on flowing injection at 35 °C. To obtain the most remarkable separation efficiency within a short time, the mobile phase, elution conditions, and other chromatographic parameters were optimized. Consequently, gradient elution was adopted using H\(_2\)O (A) and methanol (B) as a mobile phase. The isotropic elution was programmed as follows: 0–5 min, 100% B; The flow rate was 0.2 mL min\(^{-1}\), and the injection was 1.0 μL.

**MS analysis:** The HESI parameters were optimized for accurate mass measurement as follows: sheath gas flow rate, 30 L min\(^{-1}\); auxiliary gas flow rate, 8 L min\(^{-1}\); spray voltage, 3.5 kV; capillary temperature, 320 °C; nebulizer RF level, 50 V; auxiliary gas heater temperature, 120 °C; and collision energy, 35 eV. Nitrogen gas was used for spray stabilization, high energy collision dissociation, and damping gas in the C-trap. High-accuracy spectra were obtained in negative-ion mode. The mass range in the full scanning experiments was 200–3000 m/z.

**FT-IR spectroscopy analysis.** FT-IR spectra were recorded with a Perkin Elmer Spectrum One instrument (USA) and scanned between 4000 and 400 cm\(^{-1}\). KBr was mixed with the powdered samples to prepare the thin films. Materials were prepared at a concentration of 17.6 mM. KBr thin film was used as blank control.

**UV/Vis spectroscopy analysis.** UV/Vis spectra were recorded on a Shimadzu UV-2450 spectrometer (Japan). We collected information from 800 to 200 nm. Then 1 mL of the sample was detected by using a 10 mm quartz cell.

**Fluorescence spectroscopy analysis.** The samples were detected using Hitachi F-7000 (Japan) in a 1 mm quartz cell. We prepared rhein solution and hydrogel with a concentration of 14.1 mM. Samples were excited at 450 nm, monitoring the emission from 470 to 800 nm.

**X-ray powder diffraction test.** XRD patterns were carried out on a D/max 2550 power diffractometer (Japan) using a graphite-filtered Cu Ka radiation manipulating at 40 kV. The sample was rhein xerogel.

**Circular dichroism (CD) analysis.** Circular dichroism spectra of different samples were obtained by using Jasco-815. Approximately 200 μL of the sample was detected by using a 1 mm quartz cell. The bandwidth was set to 2 nm with a scanning speed of 100 nm min\(^{-1}\). All scans were taken from 200 to 600 nm, and all spectra were an average of 5 scans.

**DFT calculation.** The Gaussian 09 software package was used to perform geometry optimization and vibration analysis. The geometry optimization and vibration analyses were performed using M06-2X hybrid functional with all-electrons basis set 6-311 G*. The vibration frequencies were evaluated at the same level of theory that of the optimization step.

**In vitro drug release studies.** First, 1 mL of hydrogel sample (14.1 mM and 21.1 mM) with 1 mL of PBS buffer solution (pH 7.4, 0.01 M) was placed into separate dialysis bags (Viskase, MD25-2500, MW-2500) and incubated in 150 mL PBS buffer solution (pH 7.4, 0.01 M) at 37 °C. Then, 10 mL of sample was removed...
ELISA method. BV2 cells (1 × 10^5 cells per well in a 24-well plate) were pretreated with treatments for 1 h and stimulated with LPS (100 ng/mL). After treatment for 24 h and 48 h, the supernatants were collected. The concentrations of TNF-α, IL-1β, IL-6, IL-12, and iNOS in the culture medium were measured by ELISA kits, following the procedures recommended by the supplier (CUSABIO, Wuhan, China).

Cell viability measurement. Cells were seeded in 96-well plates (1 × 10^3 cells/well). The cells were then treated with a series of concentrations of drugs for 12 h. Then, 10 μL of MTT reagent (5 mg/mL, Sigma) was added to each well and incubated for 4 h. Afterward, the supernatants were removed. Next, 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured with a microplate reader (Huangson Technology Development Co., Ltd., Shenzhen, China).

Cell viability measurement. Cells were seeded in 96-well plates (1 × 10^3 cells/well). The cells were then treated with a series of concentrations of drugs for 12 h. Then, 10 μL of MTT reagent (5 mg/mL, Sigma) was added to each well and incubated for 4 h. Afterward, the supernatants were removed. Next, 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured with a microplate reader (Huangson Technology Development Co., Ltd., Shenzhen, China).

Immunoﬂuorescence staining. After various treatments, BV2 cells were fixed in 4% paraformaldehyde and then permeabilized with 0.3% Triton X-100. The cells were blocked with 5% bovine serum albumin (BSA). Subsequently, the cells were immunostained with primary antibodies, including TNF-α, IL-1β, IL-6, IL-12, and iNOS in the culture medium were measured by ELISA kits, following the procedures recommended by the supplier (CUSABIO, Wuhan, China).

ELISA method. BV2 cells (1 × 10^5 cells per well in a 24-well plate) were pretreated with treatments for 1 h and stimulated with LPS (100 ng/mL). After treatment for 24 h and 48 h, the supernatants were collected. The concentrations of TNF-α, IL-1β, IL-6, IL-12, and iNOS in the culture medium were measured by ELISA kits, following the procedures recommended by the supplier (CUSABIO, Wuhan, China).

Western blot assay. Total cellular and nuclear proteins were extracted from BV2 microglia cells. The protein concentrations were determined by BCA protein assay kit. Proteins were electrophoresed on 10% SDS-polyacrylamide gels, and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were blocked in 5% skim milk with 20% Tween20 (TBST) for 1 h at room temperature. Finally, nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Sigma. All images were captured using a confocal microscope (TCS SP8 X & MP, Leica, Germany).

Lamarckian Genetic Algorithm (LGA) was used and dimensions of the grids were set at 60 × 60 × 60 Å in the x, y, and z directions, with a spacing of 0.375 Å between the grid points and the center placed at the active site of TLR4-MD-2 complex crystallographic structures. All other docking and consequent scoring parameters used were kept at their default settings. The docked conformation corresponding to the lowest binding energy was selected as the most probable binding conformation.

Statistical analysis. The results for statistical analysis are presented as the mean ± standard error mean (SEM). The statistical differences between groups were tested by one-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey’s post hoc test using GraphPad Prism software (version 7.0, La Jolla, CA, USA). P-value < 0.05 was considered statistically significant (**p<0.01, ***p<0.001).

Data availability. The authors declare that all data which supports the findings are provided with the paper and its supplementary information. All data is available from the corresponding authors upon reasonable request.
24. Xu, X. et al. Rhein exhibits antioxidative effects similar to Rheubarb in a rat model of traumatic brain injury. BMC Complement. Altern. Med. 17, 140–149 (2017).
25. He, L. N. et al. Reactive metabolite activation by CYP2C19-mediated rhein hepatotoxicity. Xenobiotica 45, 361–372 (2015).
26. Wu, W., Yan, R., Yao, M., Zhan, Y. & Wang, Y. Pharmacokinetics of anthraquinones in rat plasma after oral administration of a rhubarb extract. Biomed. Chromat. 28, 564–572 (2014).
27. Gómez-Gaete, C. et al. Development, characterization and in vitro evaluation of biodegradable rhein loaded microparticles for treatment of osteoarthritis. Eur. J. Pharm. Sci. 96, 390–397 (2016).
28. Rizwanullah, M., Ahmad, J. & Amin, S. Nanostructured lipid carriers: a novel platform for chemotherapeutics. Curr. Drug Deliv. 13, 4–26 (2016).
29. Jin, H. et al. Tumor ablation and therapeutic combination by an injectable peptide hydrogel. Acta Biomater. 12, 3293–3310 (2016).
30. Lyu, D., Chen, S. & Guo, W. Liposome crosslinked polyacrylamide/DNA hydrogel: a smart controlled-release system for small molecular payloads. Small 14, 170439–170446 (2018).
31. Mealy, J. E. et al. Injectable granular hydrogels with multifunctional properties for biomedical applications. Adv. Mater. 30, 1705912–1705917 (2018).
32. Ikeda, M. et al. Installing logic-gate responses to a variety of biological substances in supramolecular hydrogel-enzyme hybrids. Nat. Chem. 6, 511–518 (2014).
33. Wang, X. et al. A Co(2+) -selective and chirality-sensitive supramolecular metallohydrogel with a nanofiber network skeleton. Nanoscale 8, 6479–6483 (2016).
34. Young, L. M. et al. Screening and classifying small-molecule inhibitors of amyloid formation using ion mobility spectrometry-mass spectrometry. Nat. Chem. 7, 73–85 (2015).
35. Yuanjiao, L. et al. Mass spectrometric and spectrophotometric analyses reveal an alternative structure and a new formation mechanism for melanin. Anal. Chem. 87, 7958–7963 (2015).
36. Ishiwata, I. et al. Gram-positive antimicrobial activity of amino acid-based hydrogels. Adv. Mater. 27, 648–654 (2015).
37. Wang, X. et al. Designing isometrical gel precursors to identify the gelation pathway for nickel-selective metallohydrogels. Dalton. T. 45, 18438–18442 (2016).
38. Sun, Z. et al. Ferrocenyl phenylalanine: a new strategy toward supramolecular hydrogels with multistimuli responsive properties. J. Am. Chem. Soc. 135, 13379–13380 (2013).
39. Yao, H., Domoto, K., Ioshashi, T. & Kimura, K. In situ detection of birefringent mesoscopic H and J aggregates of thiacyrbarocyanine dye in solution. Langmuir 21, 1067–1073 (2005).
40. Ishikawa, K., Kameta, N., Masuda, M., Asakawa, M. & Shimizu, T. Boroxine nanotubes: moisture-sensitive morphological transformation and guest release. Adv. Funct. Mater. 24, 603–609 (2014).
41. Yuan, W. Z. et al. Changing the behavior of chromophores from aggregation-caused quenching to aggregation-induced emission: development of highly efficient light emitters in the solid state. Adv. Mater. 22, 2159–2163 (2010).
42. Liu, K. et al. Coordination-triggered hierarchical folate/zinc supramolecular hydrogels leading to printable biomaterials. ACS Appl. Mater. Interfaces 10, 4530–4539 (2018).
43. Liu, G. et al. Controlling supramolecular chirality of Two-component hydrogels by J- and H-aggregation of building blocks. J. Am. Chem. Soc. 140, 6467–6473 (2018).
44. Xu, H. et al. Supramolecular nanofibers of curcumin for highly amplified radiosensitization of colorectal cancers to Ionizing radiation. Adv. Funct. Mater. 28, 1707140–1707150 (2018).
45. Hennessy, E., Griffin, E. W. & Cunningham, C. Astrocytes are primed by chronic neurodegeneration to produce exaggerated chemokine and cell infiltration responses to acute stimulation with the cytokines IL-1β and TNF-α. J. Neurosci. 35, 8411–8422 (2015).
46. Fan, H. H. et al. Hyperoside inhibits lipopolysaccharide-induced inflammatory responses in microglial cells via p38 and NF-κB pathways. Int. Immunopharmac. 50, 14–21 (2017).
47. Jung, J. S. et al. Suppression of LPS-induced neuroinflammation by morin via MAPK, PI3K/Akt, and PKA/HO-1 signaling pathway modulation. J. Agric. Food. Chem. 63, 373–382 (2016).
48. Rahimifard, M. et al. Targeting the TLR4 signaling pathway by polyphenol: A novel therapeutic strategy for neuroinflammation. Ageing Res. Rev. 36, 11–19 (2017).
49. Lu, Y. C., Yeh, W. C. & Ohashi, P. S. LPS/TLR4 signal transduction pathway. Cytokine 42, 145–151 (2008).
50. Zhu, J. et al. Involvement of mitogen-activated protein kinase and NF-κB signaling pathways in perfluorooctane sulfonic acid-induced inflammatory reaction in BV2 microglial cells. J. Appl. Toxicol. 35, 1539–1549 (2015).
51. Lan, X. et al. Pinocembrin protects hemorrhagic brain primarily by inhibiting toll-like receptor 4 and reducing M1 phenotype microglia. Brain Behav. Immun. 61, 326–339 (2017).
52. Gao, H. et al. Total tanshinones exhibits anti-inflammatory effects through blocking TLR4 dimerization via the MyD88 pathway. Cell Death Dis. 8, e3004 (2017).
53. Lan, X., Han, X., Li, Q., Yang, Q. W. & Wang, J. Modulators of microglial activation and polarization after intracerebral haemorrhage. Nat. Rev. Neuro. 13, 420–433 (2017).
54. Cui, H. & Xu, B. Supramolecular medicine. Chem. Soc. Rev. 46, 6430–6432 (2017).
55. Cheetham, A. G., Chakroun, R. W., Ma, W. & Cui, H. Self-assembling produgs. Chem. Soc. Rev. 46, 6638–6663 (2017).
56. Yanbin, C. et al. Environment-sensitive fluorescent supramolecular nanofibers for imaging applications. Anal. Chem. 86, 2193–2199 (2014).
57. Yang, C. et al. A supramolecular hydrogelator of curcumin. Chem. Commun. 50, 9413–9415 (2014).
58. Dong, J. et al. Baicalin inhibits the lethality of ricin in mice by inducing protein oligomerization. J. Biol. Chem. 290, 12899–12907 (2015).

Acknowledgements
This work was supported by the National Natural Science Foundation of China (Nos. 81303074, 81673719, 21473257, and 21773311), the Outstanding Young Foundation of Hunan Provincial Natural Science Foundation of China (No. 2019JJ30042), the Hunan Provincial Natural Science Foundation of China (No. 2019JJ30960), and the Special Program for Applied Research on Super Computation of NSFC-Guangdong Joint Fund. We are very grateful to Prof. Zhihui Yang from NanKai University for giving us guidance on experimental methods of using LC-MS to evaluate the stability of rhein gel in cells.

Author contributions
Y.W., Y.Z., R.F. and T.T. conceived and designed the research. J.Z. prepared rhein hydrogel and investigated the self-assembly mechanism of the rhein hydrogel. R.F., Y.Y., H.Y., J.L., P.Z. and P.G. conducted the study of cytotoxic and anti-inflammatory mechanisms of rhein hydrogel. H.W., T.Y. and L.D. carried out the DFT theoretical calculation. J.Z. performed the spectroscopic characterizations. L.Y. and L.R. performed mass experiments. Z.S. prepared and performed data collection. J.Z., Y.W., R.F. and Y.Y. drafted and revised the manuscript. J.Z., Y.Z. and Y.W. wrote Supplementary information.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09601-3.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Journal peer review information: Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2019