A Simple Injectable Moldable Hydrogel Assembled from Natural Glycyrrhizic Acid with Inherent Antibacterial Activity

Xia Zhao, Hao Zhang, Yuxia Gao, Yuan Lin, and Jun Hu*

ABSTRACT: Injectable low-molecular-weight hydrogels (LMWHs) from biocompatible materials have attracted much attention in biomedical applications because they can adapt any desired sizes and cavity shapes. Searching for simple, biocompatible injectable LMWHs owning inherent antibacterial activity without complicated chemical modification remains an open question to avoid the tedious synthesis/purification process and the easy bacterial infection of hydrogels in a moist environment. In this work, glycyrrhizic acid (GL), a naturally occurring compound, was found to form a stable transparent LMWH at 37 °C in physiological phosphate buffered saline (PBS) with nanoclusters as the microstructures. Moreover, this hydrogel exhibited great injectable and moldable properties. The antibacterial study showed that the growth of Gram-positive Staphylococcus aureus (S. aureus) could be completely inhibited by GL, whereas noneffect on Gram-negative Escherichia coli (E. coli) was observed. In addition, cell viability and hemolysis assay revealed that GL had good biocompatibility and hemocompatibility to mammalian cells because of its natural origin. Our simple biocompatible injectable moldable LMWH with inherent antibacterial ability has potential in the area of biomaterials and 3D bioprinting.

KEYWORDS: low-molecular-weight hydrogel, glycyrrhizic acid, natural product, injectability, antibacterial activity

INTRODUCTION

Low-molecular-weight hydrogels (LMWHs), consisting of three-dimensional networks assembled from small molecules in water, have been widely used in the area of tissue engineering, drug delivery, cell encapsulation, and wound healing over the past decades. Among them, injectable LMWHs from biocompatible materials have drawn more attention in tissue engineering applications because they can adapt any desired sizes and cavity shapes. In general, they need to fulfill some basic requirements such as hydrophilicity, biocompatibility, biodegradability, quick gel–sol–gel transition, and maintenance of mechanical strength after injecting to target sites. For example, Akiyama and co-workers have synthesized a LMWH consisting of a 16-amino acid peptide and found that it could assess the transplantation feasibility of isolated mucosal cells to repair a damaged middle ear. Wang’s group used an injected peptide solution to form a 3D LMWH in situ, which effectively filled the cavity and bridged the gaps in the wound created in the brain tissue. However, these reported works either required tedious synthesis/purification process of gelators, or ignored their antibacterial activity as hydrogels are very prone to bacterial infection in moist environment which could cause serious infections at target sites. Thus, searching for biocompatible injectable LMWHs with inherent antibacterial properties without complicated synthesis and purification process remains an open question.

As naturally occurring compounds, glycyrrhizic acid (GL, Figure 1A) is a principal component extracted from licorice roots and exhibits extensive biological activities. In addition, GL has been widely used as a food sweetener because it has 50 times the sweetening power of sucrose. In terms of chemical structure, GL consists of a hydrophobic triterpenoid aglycon (glycyrrhetinic acid, GA) and a hydrophilic diglucuronic unit linked via a glycosidic bond. Such an amphiphilic structure endows GL with good assembly capability. For example, Yang and co-workers used GL as an assembled stabilizer to transform liquid oil into soft-solid structured materials; our previous work revealed that the 1D nanofibers assembled from GL can emulsify and stabilize 80 wt % agricultural oil, thus affording the stable agricultural Pickering emulsion. Obviously, natural GL would be an ideal candidate in preparing simple injectable LMWHs with inherent antibacterial properties.

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A mixture was sonicated 2 min for the uniform dispersion and dispersed in physiological PBS (10 mM, pH 7.4) in a test tube. As a general procedure, the desired amount of GL powder was heated at 80 °C in oil-bath until a clear solution formed. Lastly, the solution was kept at 37 °C to test whether a hydrogel formed or not by the “inverted test tube method”. The results showed that GL formed a stable transparent LMWH in PBS at 37 °C when the concentration reached 1.5 mM (0.13 wt %) (Table 1, Figure 1B, inset). Its morphology was investigated by scanning electron microscopy (SEM) and transmission electron microscope (TEM). Before subjecting to SEM analysis, GL hydrogel was lyophilized under vacuum at −60 °C and coated by gold, while in the preparation of TEM samples, it was diluted 20 times and dropped on copper grid. SEM images showed that GL hydrogel had the nanocluster structures (Figure 1B,C), which was further confirmed by TEM images clearly (Figure 1D,E), with the average diameter of ∼90 nm. Because of the amphiphilic nature of GL, the hydrophobic GA moieties were sequestered within the interior and the hydrophilic diglucuronic units were projected on the outside surface, primarily promoted by the synergistic effect of hydrophobic forces and hydrogen bonding, thus resulting in the formation of nanoclusters. Consequently, these nanoclusters would further aggregate into dense network for the generation of LMWH in PBS.

To elucidate the mechanical strength and thixotropic property of GL hydrogel, rheological tests were carried out. Oscillatory frequency sweep measurements showed the values of storage modulus (G') and loss modulus (G'') were around 194 and 20 Pa, respectively, indicating the intrinsic viscoelasticity of GL hydrogel (Figure 2A). Moreover, the strain amplitude sweep was employed to determine the deformation limit. As shown in Figure 2B, the gel–sol transition occurred at the strain of 13%, implying the network destruction at such a strain. According to the results of strain sweep experiments, the thixotropic property was verified by an alternating step-strain test upon external strain as shown in Figure 2C. In step 1, the GL hydrogel was subjected to a low strain of 1% (below the deformation limit), where the G' was greater than the respective G'', indicating the hydrogel remained undamaged. In step 2, a higher strain was applied to GL hydrogel (γ = 30%), and the G' drastically decreased below the G'', which demonstrated that the cross-linked network ruptured into the sol state. In step 3, after removing the high strain and applying a low strain (γ = 1%) again, the mechanical property of GL hydrogel quickly recovered. Clearly, GL hydrogel had the good thixotropy and structural stability even after three alternating strain tests. The fast recovery of mechanical strength was apparently due to the formation of noncovalent interactions within GL gelator molecules.

Besides, GL hydrogel showed excellent injectability, and the gel volume could be controlled by injecting the hydrogel onto glass slides without blockages in the needle (Figure 2D, top). Meanwhile, its mechanical strength was visually confirmed by forming moldable hydrogels using different sized molds like pentagram, pentagon, and heart (Figure 2D, bottom). This type of injectable and moldable LMWHs has the great potential applications in 3D bioprinting.

It is known that Gram-positive S. aureus and Gram-negative E. coli are two common sources of infections requiring hospitalization. To examine the antibacterial ability of GL, a single colony of S. aureus or E. coli was inoculated in Luria–Bertani (LB) broth medium and incubated at 37 °C overnight with shaking at 200 rpm. After overnight preculture, bacterial suspensions were diluted to 1 × 10⁸ cfu mL⁻¹ in LB medium, and the cell viability assay and hemolysis assay with red blood cells revealed that GL inhibited the synthesis of DNA, RNA, and protein within the bacteria. Conversely, there was noneffect of GL on Gram-negative Escherichia coli (E. coli). In addition, the cell viability assay with red blood cells revealed that GL has great biocompatibility because of its natural origin. It is believed that our simple biocompatible injectable moldable LMWH with inherent antibacterial ability will provide an ideal candidate for biomaterials.

**RESULTS AND DISCUSSION**

As a general procedure, the desired amount of GL powder was dispersed in physiological PBS (10 mM, pH 7.4) in a test tube with the final concentration shown in Table 1. After that, the mixture was sonicated 2 min for the uniform dispersion and

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*S = solution, PG = partial gel, G = gel.

Figure 1. (A) Chemical structure of glycyrrhizic acid (GL); (B, C) SEM and (D, E) TEM images of GL hydrogel in PBS (10 mM, pH 7.4). Inset in B: digital photograph of GL hydrogel. Concentration of GL was 4 mM (0.34 wt %).
and then supplemented with GL at a final concentration of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mM (0−0.17 wt %). Lastly, the cultures were incubated at 37 °C overnight with gentle shaking. The OD600 (optical density at 600 nm) were recorded to monitor the bacterial growth and determine the minimal inhibitory concentration (MIC) (the lowest concentration that inhibits the visible growth of bacteria) of GL hydrogel. As shown in Figure 3A, the growth of S. aureus was completely inhibited by 1.5 mM (0.13 wt %) GL, whereas noneffective against the growth of E. coli (up to 2 mM, 0.17 wt %) was observed. It indicated that GL showed a strong selective effectiveness against Gram-positive S. aureus with the MIC of 1.5 mM (0.13 wt %), which might be due to the GA moiety of GL could inhibit the synthesis of DNA, RNA, and protein within the bacteria.35 Furthermore, it was subjected to time-dependent growth-inhibition assays against both S. aureus and E. coli. As shown in Figure 3B, a steady increase in OD600 of S. aureus with time was noticed when concentration was 0.5 mM (0.042 wt %) below the MIC. At the MIC of 1.5 mM (0.13 wt %), GL can completely inhibit the growth of S. aureus, showing strong antibacterial potential against Gram-positive bacteria. To be specific, E. coli treated with GL at 1.5 mM (0.13 wt %) continued growing with time, further confirming that GL was ineffective in inhibiting the activity of Gram-negative bacteria (Figure 3C). The remaining bacterial cells after treatment with GL were allowed to grow on the agar plate for recultivation. As can be seen from Figure 3D, the GL could almost completely eradicate S. aureus at the MIC of 1.5 mM (0.13 wt %), whereas the negligible effect toward E. coli was observed. We speculated that the selectivity of GL toward Gram-positive S. aureus was mainly attributed to the difference in cell wall structures between Gram-negative and Gram-positive bacteria. Compared with Gram-positive bacteria, Gram-negative bacteria have a bilayer membrane structure, which is composed of an outer lipopolysaccharides (LPS) membrane and an inner phospholipid membrane.37 Such an outer membrane with a dense layer of negatively charged LPS may act as a strong barrier to prevent the entry of GL into bacteria.

To further examine the antibacterial activities, we performed fluorescence-based live/dead assays. As shown in Figure 4A, for bacteria treated with PBS, both S. aureus and E. coli were most alive as indicated by the green fluorescence. Conversely, GL caused a dramatic increase in the number of dead S. aureus.
cells, as indicated by the dominant red fluorescence signal. Meanwhile, no obvious red fluorescence was observed in E. coli treated with GL. These results were fully consistent with the growth inhibition assays. In addition, SEM images were used to reveal the individual bacterial morphology during the treatment. As can be seen from Figure 4B, bacteria showed smooth and intact cell walls. After treatment with GL, both S. aureus and E. coli showed the similar morphology as the control group, which corresponded to reported study that the GA moiety could inhibit Gram-positive bacteria without disrupting membranes.36

For application in clinic as an antibacterial biomaterial, it is important to evaluate the cytotoxicity and hemocompatibility of GL hydrogel. Cell viability assay was first performed to investigate the effect of GL hydrogel on the viability of normal fibroblast L929 cells. Briefly, the L929 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO2 at 37 °C. The cells were harvested and seeded in 96-well plate at a seeding density of 1 × 10^4 cells per well, and incubated with GL at the concentration of 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, and 2.0 mM (0–0.17 wt %). After incubating for 24 h, the cell viability was evaluated with CellTiter-Blue cell viability assay kit (Promega). The relative cell viability was calculated by comparison to the negative control which is L929 cells treated with PBS. As shown in Figure 5A, after treatment with different concentrations of GL for 24 h, the fluorescence intensities of L929 cells were comparable with that of negative control. It indicated that GL showed negligible cytotoxicity up to the concentration of 2 mM (0.17 wt %) which was higher than the MIC for S. aureus. Moreover, cells after treatment with GL for 24 h were costained with FITC-conjugated phalloidin (for cytoskeleton) and DAPI (for nuclei). As shown in Figure 5B, GL treated cells kept the same morphology as the control cells, further confirming the biocompatibility of GL for mammalian cells.

Furthermore, a hemolysis assay with red blood cells (RBCs) was conducted to evaluate the blood compatibility. After treatment with different concentrations of GL hydrogel, intact RBCs were pelleted by centrifugation, and the amount of hemoglobin from broken RBCs released into the supernatant was measured using the UV–vis spectrum. RBCs in PBS and RBCs in water were set as a negative control and a positive control, respectively. As shown in Figure 6A, hemolysis ratios of RBCs were minimal for different concentrated GL. No obvious hemolysis was observed when the concentration was up to 2 mM (0.17 wt %) which was higher than the MIC for S.

Figure 4. (A) Representative fluorescence images of live (green) and dead (red) bacteria after treatment with GL. (B) SEM images of bacteria after treatment with GL.

Figure 5. (A) Viability of L929 fibroblast cells treated with GL; (B) morphologies of L929 cells after treatment with 2 mM (0.17 wt %) GL. Cells were stained with FITC-conjugated phal-lloidin (green) and DAPI (blue).
*S. aureus* Figure 6B showed the photographs of RBCs treated with different concentrations of GL. The supernatant of RBCs suspensions was as clear as the negative control, which was consistent with the results in Figure 6A. The corresponding optical images of RBCs showed that the RBCs did not undergo morphological transformation after being incubated with GL (Figure 6C), further confirming the negligible hemolysis. All the above results demonstrated that GL had low cytotoxicity and good hemocompatibility to mammalian cells.

### CONCLUSION

In summary, we used natural glycyrrhizic acid as a gelator to construct a stable LMWH at 37 °C in physiological PBS without additional chemical modification. This nanocluster-structured LMWH exhibited great injectable and moldable properties. Moreover, the growth of Gram-positive *S. aureus* could be completely inhibited by GL compared with Gram-negative *E. coli*. In addition, cell viability and hemolysis assays revealed that GL has low cytotoxicity and good hemocompatibility to mammalian cells because of its natural origin. Our work provides a simple, biocompatible injectable moldable LMWH with inherent antibacterial ability, which will be of great interest in the area of biomaterials and 3D bioprinting.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acsabm.9b01007](https://pubs.acs.org/doi/10.1021/acsabm.9b01007).

Materials and methods; antibacterial assay; live/dead fluorescent staining; morphological characterization of bacteria; cell viability assay; cell imaging; hemolysis assay (PDF)

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### Notes

The authors declare no competing financial interest.

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### ABBREVIATIONS

- DAPI, 4′,6-diamidino-2-phenylindole
- FITC, fluorescein isothiocyanate

### REFERENCES


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