Application of Nanoparticles Used for Gastric Ulcer Therapy: 
*In vitro and In vivo Studies (2/3)*
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中文摘要
胃潰瘍為一種普見消化系統疾病，研究得知，胃幽門螺旋桿菌寄居黏膜上皮細胞引發胃潰瘍疾病。臨床上常用抗生素來對抗胃幽門螺旋桿菌，由於使用藥物常須持續一段長時間，易導致引起服用藥物的病人會有頭暈、腹瀉、過敏等副作用和抗藥性胃幽門螺旋桿菌種種問題。同時，當胃幽門螺旋桿菌感染胃壁細胞時，所分泌毒素影響細胞間隙蛋白質結構，引起胃壁細胞嚴重發炎和胃癌危險。本研究計畫補助為製備具臨床應用性標靶性褐藻醣-幾丁聚醣/肝素奈米載體，形成一可標的胃幽門桿菌和包覆藥物能力，應用在抑制胃幽門螺旋桿菌感染細胞情況，分析奈米載體與胃幽門螺旋桿菌在細胞間相關蛋白影響。同時在動物實驗，利用胃幽門桿菌感染老鼠測試標靶性藥物奈米載體在活體藥物傳遞應用性。

關鍵詞: 褐藻醣、幾丁聚醣、胃幽門螺旋桿菌

Abstract
*Helicobacter pylori* is a significant human pathogen that recognizes specific carbohydrate receptors, such as the fucose receptor, and produces the vacuolating cytotoxin, which induces inflammatory responses and modulates the cell-cell junction integrity of the gastric epithelium. In the present study, we combined fucose-conjugated chitosan and genipin-cross-linking technologies in preparing multifunctional genipin-cross-linked fucose-chitosan/heparin nanoparticles to encapsulate amoxicillin of targeting and directly make contact with the region of microorganism on the gastric epithelium. The results show that the nanoparticles effectively reduced drug release at gastric acids and then released amoxicillin in an *H. pylori* survival situation to inhibit *H. pylori* growth and reduce disruption of the cell-cell junction protein in areas of *H. pylori* infection. Furthermore, with amoxicillin-loaded nanoparticles, a more complete *H. pylori* clearance effect was observed, and *H. pylori*-associated gastric inflammation in an infected animal model was effectively reduced.

Keywords: fucose, chitosan, *Helicobacter pylori*
2. Materials and methods

2.1. Preparation of genipin-FCS/Hep NPs

In brief, aqueous Hep (1.0 mg/mL, 2.0 mL, pH 7.4) was added by flush mixing with a pipette tip into aqueous FCS at various concentrations (0.6, 0.9, 1.2, or 1.5 mg/mL, 10.0 mL, pH 6.0). The FCS/Hep NPs produced were collected by centrifugation at 32,000 rpm for 50 min. The FCS/Hep NPs prepared with this specific composition for a suitable size distribution and zeta potential were used for the rest of the genipin-FCS/Hep NP study. Secondly, the FCS/Hep NP sample (0.5 mg/mL) was collected at composition FCS (1.2 mg/mL, 10.0 mL) to Hep (1.0 mg/mL, 2.0 mL). Then, the distinct concentration genipin solution (0, 0.125, 0.250, 0.375, and 0.500 mg/mL, 1.0 mL) was mixed into the aqueous FCS/Hep NP solution (1.0 mL) through a pipette tip with gentle stirring and was allowed to react for 2 h to form genipin-FCS/Hep NPs. The NPs produced were collected by centrifugation, and the size distribution and zeta potential were measured.

2.2. Encapsulation efficiency and release profiles of amoxicillin

To study the release profiles of amoxicillin from test NP samples (amoxicillin-loaded non-genipin-FCS/Hep NPs or genipin-FCS/Hep NPs), the amoxicillin-encapsulated NP system was prepared. The amoxicillin solution (4 mg/mL, 1 mL) was mixed with 1 mL of aqueous 2 mg/mL Hep and then added to 10 mL of aqueous 1.2 mg/mL FCS, stirred at room temperature. Then, the collected amoxicillin-loaded FCS/Hep NPs solution was mixed into a 0.375 mg/mL genipin solution and stirred as described above to form amoxicillin-loaded genipin-FCS/Hep NPs. The release profiles of amoxicillin from test samples were studied in simulated dissolution medium.

2.3. Evaluating the relationship between H. pylori and NP co-culture with AGS cells

To examine the morphology between H. pylori and the prepared NPs in co-culture with AGS cells of the surface and the cross-section in the transwell. The transwells were washed with PBS, fixed in 3.7% paraformaldehyde, rinsed twice with cacodylic acid buffer and distilled water, and dehydrated through a series of ethanol solutions (35%–100%) for 15 min each, soaked in 100% ethanol. Each sample was subjected to supercritical carbon dioxide drying and then sputter coated with 60/40 gold-palladium and visually inspected by FE-SEM.

2.4. Western blotting assay

To assay the expression affection of cell-cell junction protein between H. pylori and the prepared NPs in a co-culture with AGS cells, western blotting staining were conducted on a junctional adhesion molecule (JAM-1) (a transmembrane cell-cell junction protein). In this procedure, there were three sample conditions: Sample 1 was only H. pylori incubated with cells for 2 h, sample 2 was amoxicillin-loaded genipin-FCS/Hep NPs added to cells for 2 h, and sample 3 was H. pylori incubated with cells for 2 h, then the amoxicillin-loaded genipin-FCS/Hep NPs were introduced into the cells for 2 h. After incubation, all test samples were aspirated, and cells were washed with PBS and incubated in a growth medium for additional 22 h.

2.5. In vivo H. pylori growth inhibition study

An H. pylori infectious animal model was established according to Qian’s method (China Patent, CN 1304729A), with some modifications to determine the ability of amoxicillin solutions or amoxicillin-loaded genipin-FCS/Hep NPs to clear H. pylori in vivo. Healthy and disease-free 6-week-old male C57BL/6J mice were used for the study. After an overnight fasting, mice were inoculated with an equal amount of bacterial suspension (1.0 mL) using the intragastric gavage method. A sterile oral feeding needle was employed with each dose containing approximately 1×10⁹ CFU/mL of H. pylori. This dose was repeated once daily for 10 consecutive days. After the development of infection 1 week later, the mice were randomly divided into different groups. Each group contained six mice and received different amoxicillin formulations (30 mg/kg in the form of an amoxicillin solution or amoxicillin-loaded genipin-FCS/Hep NPs) and genipin-FCS/Hep NPs as a control once daily for 10 consecutive days. One day after the administration of the final dose, the mice were sacrificed and their stomachs removed and subjected to the following tests. Each stomach was homogenized with sterile normal saline (3 mL/stomach) from which serial dilutions were plated on blood agar plates under micro-aerophilic conditions for 5 days at 37°C. The viable bacterial count for each gastric wall was calculated by counting the number of colonies on the agar plates. Histology analysis at gastric tissue biopsy was carried out using light microscopy. Briefly, biopsies were fixed in buffered paraffin and embedded in paraffin wax. A section of about 5 μm was stained with haematoxylin and eosin to analyze the tissue inflammatory reaction and regeneration, and the stained sections were then examined at ×200 and ×1000 magnifications under a light microscope.

3. Results

3.1. Preparation of the characterization of genipin-FCS/Hep NPs

First, the FCS/Hep NPs were produced by the ionic gelation of positively charged FCS with negatively charged Hep. As shown in Table 1, the mean particle sizes of the prepared NPs were in the range of 150–210 nm, with positive zeta potentials, depending on the relative concentrations of FCS and...
were significantly reduced following the expression of JAM-1 using the western blotting.

3.4. Western blotting assay

AGS cells treated with H. pylori and the prepared amoxicillin-loaded genipin-FCS/HP NPs analyzed the expression of JAM-1 using the western blotting method. As shown in Fig. 4, JAM-1 protein levels were significantly reduced following the H. pylori infection of AGS cells at different multiplicities of infection. We also used H. pylori–infected cells and the amoxicillin-loaded genipin-FCS/HP NPs (at 0.015 mg/mL amoxicillin concentration) introduced into the cells. We found that the amount of JAM-1 present had increased from 0.64 ± 0.03 to 0.84 ± 0.04 (H. pylori infection cells at MOI of 100) and 0.38 ± 0.07 to 0.75 ± 0.06 (H. pylori infection cells at MOI of 300), compared to the control values, respectively. As a result, we know that the prepared amoxicillin-loaded genipin-FCS/HP NPs could infiltrate and contact with H. pylori of the cell-cell junction and that their instability may release amoxicillin to inhibit H. pylori growth and then reduce the disruption of the cell-cell junction protein of the gastric epithelium.

3.5. In vivo H. pylori growth inhibition study

Figure 5 shows the in vivo clearance data of H. pylori infection. The mean bacterial count of the control group of mice that were given genipin-FCS/HP NPs (without amoxicillin) was 521.5 ± 83.8 (CFU/stomach). A treatment of a 30 mg/kg amoxicillin solution alone gave a mean bacterial count of 278.3 ± 31.5 (CFU/stomach). On the other hand, a treatment of amoxicillin-loaded genipin-FCS/HP NPs (30 mg/kg amoxicillin) gave a mean bacterial count of 75.8 ± 18.5 (CFU/stomach), with significantly increased inhibitory effects on H. pylori–infected mice compared with the amoxicillin solution alone. Meanwhile, we used a rapid urease test, also known as the Campylobacter-like organism test, which was developed by Marahall and specifically designed to detect H. pylori. In the presence of H. pylori urease, urea is converted into ammonium hydroxide and changes the color of the indicator from yellow to red. Gastric tissue biopsy was stained with hematoxylin and eosin for histological examination. Figure 6 shows the histological results of the inflammation of H. pylori-infected mice treated with genipin-FCS/HP NPs. The inflammation treated with amoxicillin solution alone was more severe than that treated with the amoxicillin-loaded genipin-FCS/HP NPs.

4. Conclusions

A multifunctional NP system of targeting H. pylori was successfully produced to directly make contact with the region of microorganism on the gastric epithelium. Our results indicate that the genipin-cross-linking FCS/HP NP–encapsulated amoxicillin could reduce drug release effect at gastric acids and could then release amoxicillin in an H. pylori survival situation to inhibit H. pylori growth and reduce the disruption of transmembrane cell-cell junction protein. Our in vivo results clearly indicate that the amoxicillin-loaded genipin-FCS/HP NPs have a more complete H. pylori clearance effect and are effective in reducing H. pylori–associated gastric inflammation phenomenon in H. pylori–infected animal models.
5. Reference


Table 1. Effects of concentrations of FCS on the particle sizes and zeta potential values of the prepared FCS/Hep NPs (n = 5).

<table>
<thead>
<tr>
<th>FCS Conc.</th>
<th>Hep Conc.</th>
<th>Size (nm)</th>
<th>Zeta (mV)</th>
</tr>
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<tbody>
<tr>
<td>0.6</td>
<td>1.0</td>
<td>156.9 ± 3.8</td>
<td>23.6 ± 3.9</td>
</tr>
<tr>
<td>0.9</td>
<td>1.0</td>
<td>168.1 ± 4.9</td>
<td>25.2 ± 1.1</td>
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<tr>
<td>1.2</td>
<td>1.0</td>
<td>184.7 ± 7.1</td>
<td>29.1 ± 0.2</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>208.7 ± 11.4</td>
<td>32.8 ± 1.1</td>
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</table>

Figure 1. Particle size distribution of prepared NPs at different genipin concentrations.

Figure 2. Amoxicillin release profiles from NPs.

Figure 3. SEM micrographs of the amoxicillin-loaded genipin-FCS/Hep NP treatment with *H. pylori* on transwell for 2 h.

Figure 4. Western blot analysis for JAM-1 of AGS cell infected with *H. pylori* and incubated amoxicillin-loaded genipin-FCS/Hep nanoparticles

Figure 5. Effects of amoxicillin solution alone and with or without amoxicillin-loaded genipin-FCS/Hep NPs in an *H. pylori*–induced gastric infection mouse model.

Figure 6. Histological image analysis of *H. pylori*–infected mouse treated with amoxicillin solution alone and with or without amoxicillin-loaded genipin-FCS/Hep NPs after.