藥物奈米載體於治療胃潰瘍之應用：體外與體內實驗 (1/3)

Application of Nanoparticles Used for Gastric Ulcer Therapy: *In vitro and In vivo* Studies (1/3)

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林宥欣 Yu-Hsin Lin[1*], 陳紫柔 Zih-Rou Chen[1], 陳浩雲 Hao-Yun Chen[1]

[1] 中國醫藥大學生物科技系

[1] Department of Biological Science and Technology, China Medical University, Taichung, Taiwan

*Corresponding Email: ylhsin@mail.cmu.edu.tw

中文摘要

消化性潰瘍為一種普見的消化系統疾病，以胃幽門螺旋桿菌 (*Helicobacter pylori；H.P)*感染情況最嚴重。臨床上對抗胃幽門螺旋桿菌以抗生素藥物(amoxicillin、clarithromycin 和 tetracycline 等)為主，但此藥物易受到胃酸的破壞而失去藥物療效，需長時間服用以達到消滅胃幽門螺旋桿菌，常造成病患的不便性。本研究希望結合奈米技術在消化性潰瘍的治療，探討包覆抗生素的奈米微粒載體對消滅胃幽門螺旋桿菌之效果與影響。實驗中將以幾丁聚醣(chitosan)與肝素(heparin)為材料，利用油/水乳化方式製備一可攜帶抗生素藥物的奈米載體(nanoparticles)，對其粒徑分佈、表面電荷、形態分析和藥物包覆率與釋放進行測試，同時，將包覆抗生素之奈米微粒載體與胃幽門螺旋桿菌進行消滅情況分析，未來期望能藉由服用包覆抗生素之奈米微粒載體降低病人服用抗生素之次數。

關鍵詞：乳化、奈米載體、胃幽門螺旋桿菌

Abstract

Gastric ulcer disease represents a worldwide health problem because of its high morbidity and mortality. *Helicobacter pylori* is considered to be an important etiological factor in gastric ulcer disease and has been suggested as a cause of gastric carcinoma. Moreover, *H. pylori* colonizes the human gastric mucus layer and adheres to the surface epithelial cells by virtue of a variety of adhesin-like proteins. In order to effectively eradicate *H. pylori* infection, the therapeutic agent must be able to penetrate through the gastric mucus layer and maintain a concentration sufficient for antibacterial activity at the infected site for a suitable length of time. The most widely recommended regimen includes a triple therapy which combines various antibiotics administered over a period of two weeks. However, the occurrence of unpleasant side effects, such as a metallic taste in the mouth, diarrhea and nausea, may cause the patient to interrupt the prescribed course of antibiotics, thus promoting the development of bacterial resistance. In the study, a platform technology in developing anti-*H. pylori* drug incorporated in nanoparticles composed of positive and negative charged polymers is proposed for eradication of targeting *H. pylori*. The first year project had developed a nanocarrier system with the ability to carry amoxicillin to increase the efficacy of amoxicillin against *H. pylori*. We used a water–in–oil emulsification system to prepare a positively charged nanoemulsion particle, then their particle size of the prepared nanoemulsion particle can be controlled by their constituted compositions and the morphology of the nanoemulsion particles was spherical in shape. *In vitro* analysis of amoxicillin release indicated that the nanocarrier system was able to control amoxicillin release in gastrointestinal dissolution medium and that amoxicillin-loaded nanoemulsion particles could localize to the site of *H. pylori* infection.

Keywords: Emulsification, Nanoparticles, *Helicobacter pylori*

Introduction

Helicobacter pylori, a Gram-negative, microaerophilic spiral bacterium that colonizes the mucosa of the human stomach, has been considered to be an important etiological factor in the development of peptic ulcer disease and gastric cancer [1–3]. For effective *H. pylori* eradication, the standard treatment in the case is a combination of drugs, including antibiotics and a proton pump inhibitor [4]. The microorganism mainly produce virulence factors, including vacuolating cytotoxin which causes epithelium cells degradation and colonized deeply within the gastric mucus layer [5,6]. The particulate system for translocation permeability through gastrointestinal mucin was found to be increased as the particle size decreased to nanosize particles [7,8]. Encapsulation of low molecular weight hydrophilic drugs such as antibiotics in these nanocarriers failed because such small and hydrophilic molecules rapidly leaked from their, nanocarriers has resulted in poor encapsulation efficiency and fast release upon dilution [9,10]. To overcome this issue, we established the water-in-oil emulsification technique for preparing nanoemulsion particles with a better encapsulated antibiotic ability. The emulsion technique is a heterogeneous system, that consists of a water phase dispersed in an oil phase in the presence of blends of hydrophilic and hydrophobic surfactants under homogenization to produce fine droplets [11]. The

efficiency of blends of hydrophilic and lipophilic surfactants between oil and water phase was primarily determined by a hydrophilic-lipophilic balance (HLB) system. A surfactant with a higher HLB value is usually more hydrophilic, and one with a lower value is more lipophilic. An optimal HLB value of the surfactants was a key factor for formation of emulsion with minimal droplets [12,13].

In our emulsion method, we selected liquid paraffin as the oil phase and two surfactants, sorbitan monolaurate (Span 20) and polyoxyethylene sorbitan monolaurate (Tween 20), to optimize surfactant levels and HLB value of the antibiotic-loaded chitosan/heparin nanoemulsion particles. Bayindir *et al*. previously developed niosomal formulations using nonionic surfactants (such as Span 20 and Tween 20) to achieve gastrointestinal stability for paclitaxel oral delivery [14]. It has also been reported that Span 20/Tween 20 mixtures increased the stability of the emulsification procedure compared with pure Span or Tween systems [15]. We prepared nanoemulsion particles composed of chitosan, heparin and an antibiotic (amoxicillin), with liquid paraffin and nonionic surfactant mixture (Span 20/Tween 20) as the oil phase. The molecular formula of hydrophilic amoxicillin is $C_{16}H_{19}N_3O_5S$-$3H_2O$ and the molecular weight (MW) is 419.45. The antibiotic amoxicillin is a semisynthetic antibiotic that binds to penicillin-binding proteins and interferes with bacterial cell wall synthesis, resulting in lysis of replicating bacteria [16,17]. It was reported that the failure of antibiotic therapies is attributable to the poor stability of the drug in the gastric acid and the poor permeation of the antibiotic across the mucus layer. This followed by resecretion into the lumen, where a sufficient amount of the drug diffuses into the bacteria [18,19].

Thus, the hypothesis of our prepare nanoemulsion particles could encapsulate amoxicillin and infiltrate into the mucus layer, subsequently, amoxicillin release from amoxicillin-loaded nanoemulsion particles, then directly acts locally on *H. pylori* at a bactericidal concentration [Fig. 1(A)]. We examined their physicochemical characteristics using fourier-transformed infrared spectroscopy (FT–IR), transmission electron microscopy (TEM), and dynamic light scattering. We also investigated amoxicillin release characteristics from the prepared nanoemulsion particles and examined the inhibition of *H. pylori* growth. In addition, the effect of the nanoemulsion particles and their mechanism of interaction with *H. pylori* were investigated in the human gastric mucosal AGS cell line (human gastric adenocarcinoma cell line) with confocal laser scanning microscopy (CLSM) [20].

Materials and Methods

2.1. Materials

Chitosan (MW 50 kDa) with approximately 85% deacetylation was obtained from Koyo Chemical Co. Ltd. (Japan). Heparin (5000 IU/mL, MW 15 kDa, 179 IU/mg) was purchased from Leo Chemical Factory (Ballerup, Denmark). Liquid paraffin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tween 20, Span 20, amoxicillin, acetic acid, 3-(4,5-dimethyl-thiazol-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), fluoresceinamine isomer I (FA), phosphate buffered saline (PBS), sodium acetate, paraformaldehyde, bismuth subnitrate, Hanks' balanced salt solution (HBSS), and β-cyclodextrin were purchased from Sigma–Aldrich (St Louis, MO). RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-ethylene diamine tetraacetic acid (trypsin-EDTA) were from Gibco (Grand Island, NY). N-hydroxy-succinimide (NHS)-functionalized cyanine 3 (Cy3-NHS) was from Amersham Biosciences (Piscataway, NJ). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine-5,5'-disulfonic acid (DilC18(5)-DS) lipophilic dye was from Molecular Probes (Eugene, USA). All other chemicals and reagents were of analytical grade.

2.2. Preparation of chitosan/heparin nanoemulsion particles by water-in-oil emulsification

The nanoemulsion particles were prepared by water-in-oil emulsification technique through homogenization using a laboratory-type rotating blade homogenizer (IKA Labortechnik, Staufen, Germany). First, the distinct Span 20:Tween 20 ratio (50:50, 67:33, 75:25, and 80:20) of the Span 20/Tween 20 mixture surfactant (0.4, 0.6, 0.8, 1.0, and 1.2 mL) was added to liquid paraffin (80.0 mL) under continuous mixing. Second, the aqueous heparin (0.2 mg/mL, 4 mL, pH 7.4) was adding to liquid paraffin containing the Span 20/Tween 20 surfactant and homogenization at 15,000 rpm at 4℃ for 2 min. Then, aqueous chitosan (0.6 mg/mL, 4 mL, pH 6.0) was slowly dropped into the resultant heparin emulsion and subject to homogenization at 15,000 rpm at 4℃ for 2 min. The prepared chitosan/heparin nanoemulsion particles were centrifuged twice at 32,000 rpm for 30 min, then the pellets were washed with distilled water and 50% aqueous alcohol to remove traces of paraffin oil and surfactant. Finally, the pellets were collected and suspended in deionized water for further study.

2.3. Characterization of prepared nanoemulsion particles

The size distribution and zeta potential value of the nanoemulsion particles at pH 1.2 (0.1 M HCl, simulated gastric medium), pH 6.0, and pH 7.0 (10 mM PBS) simulating the gastric mucosa and the *H. pylori* survival situation medium were measured using

a dynamic light scattering analyzer (Zetasizer ZS90, Malvern Instruments Ltd., UK) [21,22]. The peak variations of the nanoemulsion particles at different pH values were characterized using FT–IR (Shimadzu Scientific Instruments, USA). The morphology of the nanoemulsion particles was visualized under TEM at different pH values. The TEM samples were prepared as follows. The particle suspension was placed onto a 400 mesh copper grid coated with carbon. About 2 min after deposition, the grid was tapped with a filter paper to remove surface water and positively stained with an alkaline bismuth solution.

2.4. Encapsulation efficiency and release profiles of amoxicillin–loaded nanoemulsion particles

To study the release profiles of amoxicillin from amoxicillin-loaded chitosan/heparin nanoemulsion particles, the amoxicillin–loaded nanoemulsion particles were prepared. Amoxicillin (0.2 mg/mL, 0.4 mg/mL, and 0.6 mg/mL, 2 mL, pH 7.4) was mixed with aqueous heparin (0.4 mg/mL, 2 mL, pH 7.4) under continuous stirring for 12 hr at 4℃ and then added to liquid paraffin containing the Span 20/Tween 20 mixture surfactant with homogenization. Then, the aqueous chitosan (0.6 mg/mL, 4 mL, pH 6.0) was slowly dropped into the resultant heparin/amoxicillin emulsion during homogenization at 15,000 rpm at 4℃ for 2 min. Finally, the amoxicillin-loaded chitosan/heparin nanoemulsion particles were obtained after centrifugation, washed, and then suspended in deionized water. To determine the loading efficiency and loading content, the amoxicillin concentration was assayed by high–performance liquid chromatography (HPLC). The release profiles of amoxicillin from test particles were investigated in simulated dissolution medium (pH 1.2 for 120 min, pH 6.0 for 120 min, and pH 7.0 for 360 min) at 37℃. At set time intervals, samples were removed and centrifuged, and the supernatants were subjected to HPLC. The percentage of cumulative amount of released amoxicillin was determined using a standard calibration curve.

2.5. Viability of AGS cells treated with chitosan/heparin nanoemulsion particles

The AGS cell line (ATCC CRL 1739) was obtained from the American Type Culture Collection (ATCC). The AGS cells were cultured in RPMI 1640 medium containing 10% FBS, penicillin (100 IU/mL), and streptomycin (100 mg/mL), and were kept in an incubator at 37℃, 95% humidity, and 5% CO_2 [23]. The cells were harvested for subculture every 3 days with 0.25% trypsin plus 0.05% EDTA solution and were used for the cytotoxicity experiments. The cytotoxicity of the test samples was evaluated *in vitro* using the MTT assay. AGS cells were seeded at 5×10^4 cells/well in 96-well plates and allowed to adhere overnight. The growth medium was replaced with HBSS solution (pH 6.0) that contained various concentrations (0.02-0.20 mg/mL) of chitosan/heparin nanoemulsion particles and incubated for 2 hr. After 2 hr, the test samples were aspirated and the cells were washed twice with 100 μL of PBS. The cells were then incubated in growth medium for an additional 22 hr. The cells were then incubated in growth medium containing 1 mg/mL MTT for an additional 4 hr. Dimethyl sulfoxide (100 μL) was added to each well to ensure solubilization of the formazan crystals. The optical density was read with a Molecular Devices SpectraMax M2^e microplate spectrofluorometer (Sunnyvale, CA) at a wavelength of 570 nm. All experiments were performed six times with eight replicate wells for every sample and control per assay.

2.6. *In vitro* cellular uptake and CLSM visualization

To track the cellular particles, fluorescent Cy3-chitosan and FA-heparin were prepared according to the procedure described.The fluorescent Cy3-chitosan/FA-heparin nanoemulsion particles were prepared according to the procedure described in Section 2.2. The AGS cells were seeded onto 6-cm petri dishes at a density of 5×10^5 cells/cm^2 and incubated for 2 days. After incubation, the medium was removed and cells were treated with HBSS solution (pH 6.0) containing Cy3-chitosan/FA-heparin nanoemulsion particles at a concentration of 0.1 mg/mL. After 2 hr, the test samples were aspirated and the cells were then washed three times with PBS before being fixed in 3.7% paraformaldehyde. The cells were washed three times with PBS and stained with DAPI, which specifically bind to the nuclei. The stained cells were examined with excitation at 340, 488, and 543 nm using a CLSM.

2.7. Evaluating the relationship between H. pylori and amoxicillin-loaded nanoemulsion particles in co-culture with AGS cells

To observe the adhesion of *H. pylori* to cells, the fluorescent bacteria were labeled with DilC18(5)-DS fluorescently labeled lipophilic dye according to the procedure described in our previous study [24]. Additionally, synthesis of FA-amoxicillin was based on the reaction between the amine group of FA and the carboxylic acid group of amoxicillin. Amoxicillin (60 mg) was dissolved completely in 30 mL of deionized water and 2 mg of FA was dissolved completely in 1 mL of acetonitrile. The FA solution was added gradually to the amoxicillin solution, then 1 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was added with continuous stirring at room temperature for 120 min and then freeze dried. To remove the unconjugated FA, the dry FA-amoxicillin sample was precipitated by adding acetonitrile (30 mL), and the precipitate was subjected to repeated cycles of washing and centrifugation until no fluorescence was detected in

the supernatant. The resultant FA-amoxicillin was dissolved in deionized water then freeze dried.

To examine the effects on *H. pylori* of amoxicillin–loaded nanoemulsion particles in co-culture with AGS cells, the AGS cells were seeded on Costar Transwell six-well plates (Corning Costar Corp., NY) at a seeding density of 5×10^5 cells/insert. The AGS cell culture medium was added to both the donor and acceptor compartments. The medium was replaced every 48 hr for the first 6 days and every 24 hr thereafter. The cultures were kept in an incubator and were used for the *H. pylori* infection experiments 26–30 days after seeding [25,26]. DiIC18(5)-*H. pylori* was incubated with the AGS cells for 2 hr. The medium (pH 6.0) containing the FA-amoxicillin–loaded Cy3-chitosan/heparin nanoemulsion particles or fluorescent FA-amoxicillin solution were then introduced into the donor compartment of the AGS cells for 2 hr at 37℃. After incubation, the test samples were aspirated. The cells were washed twice with PBS and stained with DAPI for 15 min. The stained cells were examined by CLSM with excitation at 340, 488, 543, and 633 nm at 0.2 μm intervals, and 3D images were created using LCS Lite software.

Results and Discussion

3.1. Preparation of nanoemulsion particles

The Chitosan/heparin nanoemulsion particles were prepared by a water–in–oil emulsification method using a mixture of Span 20 and Tween 20 nonionic surfactants in liquid paraffin. As the amount of Span20/Tween20 mixture surfactant incorporated in emulsification phase was increased, the size of the resulting particles decreased appreciably and to give a uniform matrix with a spherical shape [Fig. 1(B)]. From these results, optimal conditions for preparing a fine chitosan/heparin nanoemulsion particles by water–in–oil emulsification were: an aqueous phase (4 mL) chitosan (0.6 mg/mL) and heparin (0.2 mg/mL), and an oil phase of paraffin oil (80 mL) containing 1.2 mL of a Span20:Tween20 in a 75:25 ratio. Nanoemulsion particles prepared to this specific composition were used for the remainder of the study.

3.2. Characterization of prepared nanoemulsion particles at specific pH values

The morphologies of the prepared chitosan/heparin nanoemulsion particles at various pH values were examined by TEM, and pH related chemical changes were followed by FT–IR [Fig. 2]. At pH 6.0 (simulating the environment of the gastric mucosa, and ideal conditions for *H. pylori* survival situation), the characteristic peaks observed at 1532 cm^{-1} and 1633 cm^{-1} were the protonated amino group ($–NH_3^+$) on chitosan and the carboxylic ions ($–COO^–$) on heparin, respectively [Fig. 2(A)]. Thus ionized chitosan and heparin formed a polyelectrolyte complex with a spherical matrix structure [Fig. 2(B)]. At pH 1.2 (simulating the pH of gastric acid), the characteristic peak observed at 1239 cm^{-1} was attributed to the heparin sulfate ions ($–SO_4^–$), but some of the $–COO^–$ groups on heparin also became protonated (–COOH, 1736 cm^{-1}). Electrostatic interactions between chitosan and heparin were therefore weaker at pH 1.2 than they were at pH 6.0, leading to greater particle sizes (289.1 ± 5.6 nm). At pH 7.0, chitosan ammonium groups were deprotonated [indicated by disappearance of the characteristic peak for $–NH_3^+$ on chitosan; Fig. 2(A)], leading to the collapse of the nanoparticles [Fig. 2(B)].

3.3. Encapsulation efficiency and release profile of amoxicillin-loaded nanoemulsion particles

We used chitosan/heparin nanoemulsion particles to encapsulate amoxicillin at various concentrations. It was shown that for an amoxicillin concentration of 0.6 mg/mL, particle size is 296.5 ± 6.3 nm and zeta potential is 29.8 ± 3.1 mV, while the amoxicillin loading efficiency and percentage loading content are 54.3 ± 2.8% and 19.2 ± 1.2%, respectively. Under optimal conditions, these amoxicillin-loaded nanoemulsion particles remained spherical [Fig. 2(B)]. We used the optimal amoxicillin concentration of 0.6 mg/mL for subsequent *in vitro* studies. Fig. 2(C) shows the *in vitro* release of amoxicillin in solutions buffered pH 1.2, 6.0, and 7.0. At pH 1.2, the nanoemulsions released 20.5 ± 1.2% amoxicillin over 120 min. At pH 6.0, the structure and morphology of nanoparticle emulsion was stable. By contrast, at pH 7.0, the prepared emulsion particles became unstable and collapsed, causing rapid release of the amoxicillin. These findings suggest that chitosan/heparin nanoemulsion particles have potential for sustained drug release.

3.4. Viability and cellular uptake of cells treated with chitosan/heparin nanoemulsion particles

We evaluated the cytotoxicity of various concentrations of chitosan/heparin nanoemulsion particles using AGS cells and MTT assay (Fig. 3). Cell viability was generally unaffected at concentrations less than 0.14 mg/mL. Thereafter, we used emulsion particles at 0.1 mg/mL to track cellular internalization of particles without causing damage to cultured cells. We used fluorescent nanoemulsion particles [Cy3-chitosan (red)/FA-heparin (green) nanoemulsion particles] to demonstrate cellular uptake. Fig. 4 shows the fluorescence intensity of those AGS cells that internalized fluorescent particles. Fluorescent signals appeared in the intercellular spaces and cell cytoplasm [superimposed red (Cy3-chitosan)/green (FA-heparin) spots; i.e., yellow spots, white arrows; Fig. 4] after 2 hr incubation with nanoemulsion particles at pH 6.0.

3.5. Relationship between *H. pylori* and

amoxicillin–loaded nanoemulsion particles

To study the interaction of amoxicillin–loaded nanoemulsion particles with *H. pylori* attached to the epithelia, we developed a process whereby fluorescent DiIC18 (5)-*H. pylori* infected monolayers of AGS cells, and the infected cells were then treated with fluorescent FA-amoxicillin alone, or FA-amoxicillin loaded in Cy3-chitosan/heparin nanoemulsion particles. As shown in Fig. 4, the fluorescent images (those after 3D reconstruction) of AGS cell monolayers were observed (the *XY* plane) and appeared at a deep level (the *XZ* plane). The AGS–adapted DiIC18 (5)-labeled *H. pylori* (purple spot) preferentially targeted cell-cell junctions and was present within the cells. The AGS cell monolayers were incubated with fluorescent FA-amoxicillin loaded in Cy3-chitosan/heparin nanoemulsion particles (Cy3-chitosan: red spot, FA-amoxicillin: green spot) and observed by CLSM to ascertain whether the nanoparticles and drug were attached in the same way to the intercellular spaces and the cell cytoplasm. The superimposed images (3D reconstruction of the *XY* and *XZ* plane), show that administered amoxicillin loaded chitosan/heparin nanoemulsion particles co-localized and interacted at the same location of intercellular spaces and the cell cytoplasm of *H. pylori* infectious sites (superimposed red spot: Cy3-chitosan, green spot: FA-amoxicillin, and purple spot: DiIC18 (5)-*H. pylori* appearing as white spots, green arrows; Fig. 5). By contrast, cells incubated with FA-amoxicillin solution alone (green spot), showed less obvious fluorescent signals in intercellular spaces than those seen after incubation with fluorescent FA-amoxicillin loaded in Cy3-chitosan/heparin nanoemulsion particles. The amoxicillin-loaded particles produced an intense fluorescence that emanated from deep within the cells, indicating that the nanoemulsion particles were capable of carrying amoxicillin to the *H. pylori* infection site. This result suggests that our nanoemulsion particles prepared had a specific interaction with AGS cell monolayers infected with *H. pylori.*

3.6. *H. pylori* growth inhibition study

Fig. 6 shows the influence on gastric mucosal cell viability of *H. pylori* infection alone and after pretreatment with amoxicillin-loaded chitosan/heparin nanoemulsion particles. The figure shows a significant reduction in AGS cell viability after infection with *H. pylori* (8×10^8 CFU/mL) alone; cell viability was 47.6 ± 2.9% that of uninfected controls. We determined the percentage of *H. pylori*-infected AGS cells after 24 hr treatment using various amoxicillin concentrations (0.0, 2.0, 4.0, 8.0, and 16.0 by mg/L). Cell survival rates seen with amoxicillin solution alone was 52.3 ± 2.4% (amoxicillin concentration of 2.0 mg/L), 57.1 ± 2.2% (4.0 mg/mL), 63.9 ± 3.9% (8.0 mg/mL), and 70.4 ± 3.5% (16.0 mg/L). Meanwhile, the cell survival rates with amoxicillin-loaded chitosan/heparin nanoemulsion particles were 65.1 ± 2.9% (at amoxicillin concentration of 2.0 mg/L), 72.6 ± 3.2% (4.0 mg/mL), 81.4 ± 4.3% (8.0 mg/mL), and 90.5 ± 3.8% (16.0 mg/mL), compared with control (without sample). Therefore, our prepared amoxicillin-loaded chitosan/heparin nanoemulsion particles with a positive surface charge significantly increased the inhibitory effects on *H. pylori* infected cells compared with amoxicillin solution alone ($p < 0.05$).

Conclusions

We prepared chitosan/heparin nanoemulsion particles using a water–in–oil emulsification system for delivery of amoxicillin to treat *H. pylori* infection. The nanoemulsion particles were stable at pH 1.2. *In vitro* drug release analysis of the nanoemulsion particles indicated that the system could control amoxicillin release in a simulated gastrointestinal dissolution medium. The amoxicillin-loaded chitosan/heparin nanoemulsion particles could localize at intercellular spaces or in the cell cytoplasm, the site of *H. pylori* infection, and significantly increased *H. pylori* growth inhibition compared with amoxicillin alone

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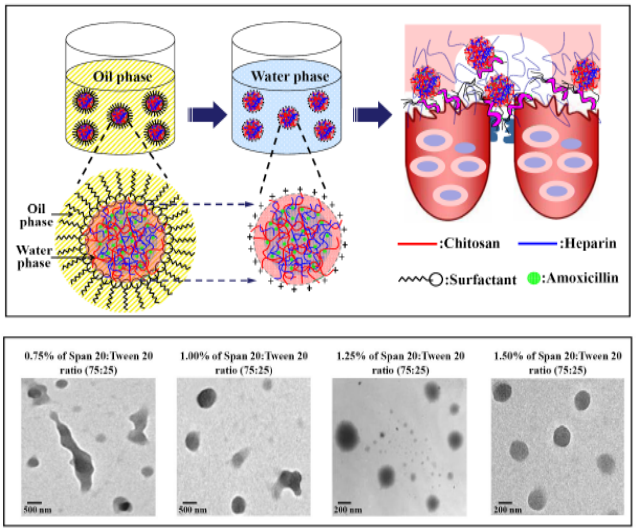

Fig. 1. (A) A representation for prepared amoxicillin loaded in chitosan/heparin nanoemulsion particles and the strategy for eradicating *H. pylori* using the nanoemulsion particles. (B) TEM micrographs of the nanoemulsion nanoparticles at different Span 20/Tween 20 mixture surfactant compositions.

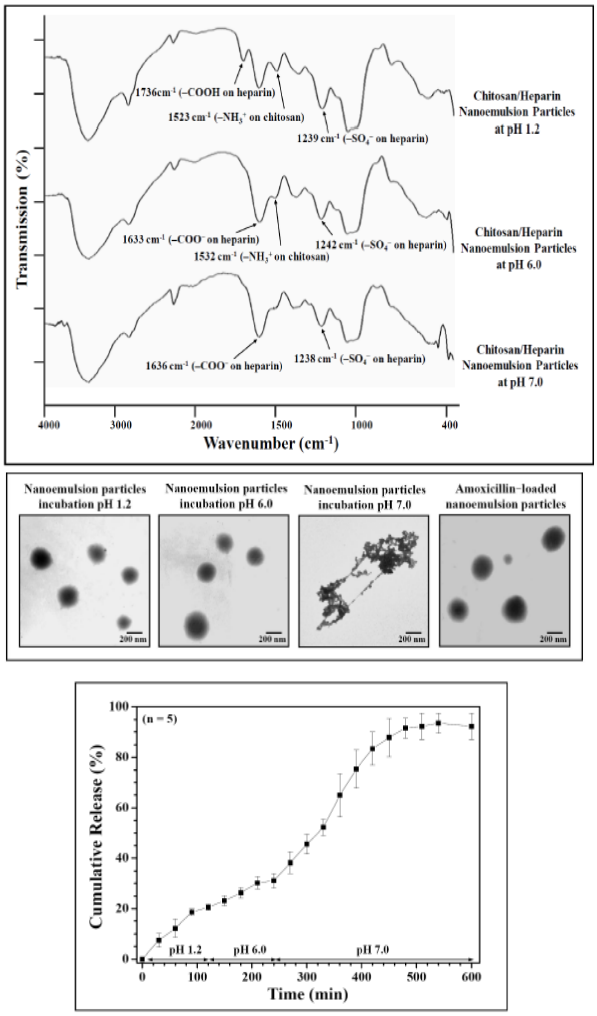

Fig. 2. (A) FT-IR spectra of chitosan/heparin nanoemulsion particles in specific pH environments. (B) TEM micrographs of the prepared nanoemulsion nanoparticles in specific pH environments. (C) *In vitro* release profiles of amoxicillin from amoxicillin-loaded nanoemulsion particles at different pH values at 37℃ (n = 5).

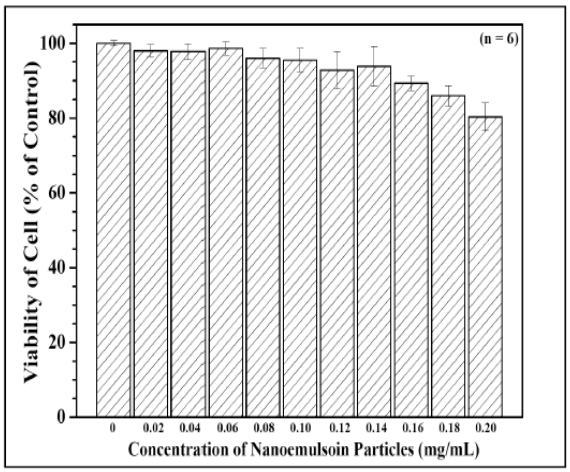

Fig. 3. Cell viability after treatment with different concentrations of chitosan/heparin nanoemulsion particles (n = 6).

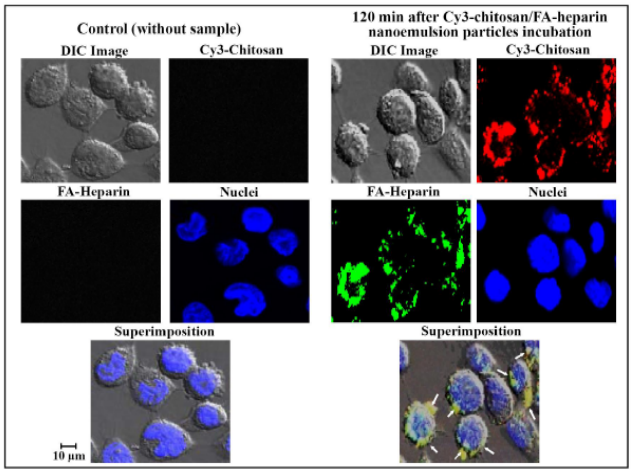

Fig. 4. Confocal images of AGS cells incubated with internalized Cy3-chitosan/FA-heparin nanoemulsion particles for 120 min.

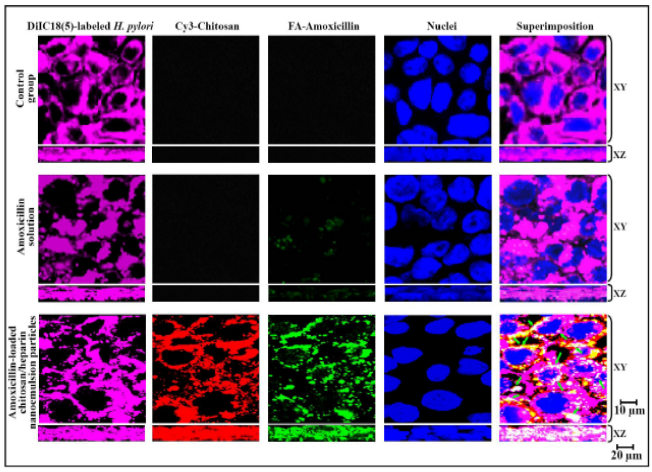

Fig. 5. Fluorescent images (after 3D reconstruction) of AGS cell monolayers infected with fluorescent *H. pylori* (DilC18(5)-labeled *H. pylori*) and incubated with FA-amoxicillin solution alone or FA-amoxicillin-loaded fluorescent Cy3-chitosan/heparin nanoemulsion particles for 2 hr.

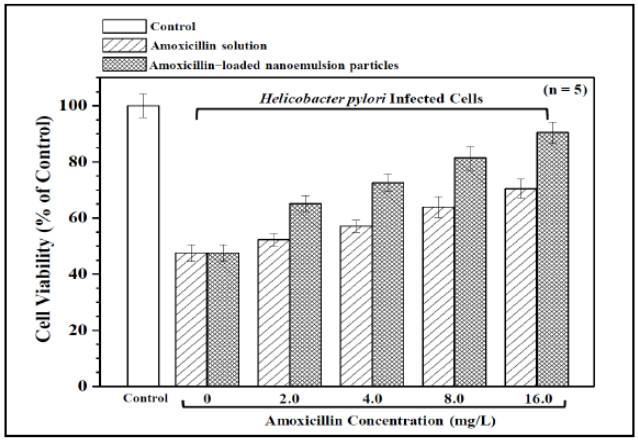

Fig. 6. Changes in cell growth after AGS cells were pre-infected with *H. pyloi* for 2 hr and then incubated with amoxicillin solution alone or amoxicillin-loaded chitosan/heparin nanoemulsion particles.