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Ethyl cellulose nanoparticles: Clarithomycin encapsulation and eradication of *H. pylori*

Porntip Pan-In[a], Wijit Banlunara[b], Nuntaree Chaichanawongsaroj[c], Supason Wanichwecharungruang[d,e,*]

[a] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[c] Innovation Center for Research and Development of Medical Diagnostic Technology Project, Department of Transfusion Medicine, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[d] Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[e] Nanotec-CU Center of Excellence on Food and Agriculture, Bangkok 10330, Thailand

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ABSTRACT

The extreme acidic environment of the stomach, its regular voidance of contents and the restricted access to the mucus covered habitat combined with the antibiotic resistance of the bacteria, all contribute to the poor success in the treatment of *Helicobacter pylori* gastric infections. Here, we demonstrate that by encapsulating clarithromycin into ethyl cellulose (EC) nanoparticles, the efficiency of *H. pylori* clearance in C57BL/6 mice infected with these bacteria was significantly improved. Clarithomycin-loaded EC nanoparticles were prepared *via* a simple yet effective anti-solvent particle induction method, to yield sub-micron sized particles with 22.3 ± 0.17% (w/w) clarithromycin loading at 86 ± 0.5% (w/w) encapsulation efficiency. The particles dispersed well in water and simulated gastric fluid and gave a minimum inhibitory concentration of 0.09–0.18 μg/ml against four strains of *H. pylori*. Encapsulation into EC particles not only enhanced the anti-adhesion activity of clarithromycin when tested with *H. pylori* and Hep-2 cells, but also gave significant enhancement of *H. pylori* clearance in the stomach of C57BL/6 mice infected with the bacteria.

1. Introduction

Helicobacter pylori, a prevalent human-specific pathogen, was discovered in 1982, confirmed as a pathogen at the end of the 1980s and classified as type I carcinogen in 1994 by the World Health Organization (WHO). At present, infection with *H. pylori* is one of the most widespread chronic bacterial infections in the world. Although the majority of *H. pylori*-infected patients will remain asymptomatic throughout life, about 10–15% patients will develop gastric diseases, such as gastritis, peptic ulcerations, gastric cancer and MALT lymphoma (Suerbaum & Michetti, 2002). Case reports, small pilot studies, and *in vitro* data have also suggested that various extra gastric diseases, *e.g.* ischemic heart disease, idiopathic thrombocytopenic purpura, iron-deficiency anemia and Alzheimer's disease, can be linked to *H. pylori* infection (Annibale, Capurso, & Delle Fave, 2002; Franceschi & Gasbarrini, 2007; Ge & Sun, 2011; Ge et al., 2011).

The stomach is the principal organ of *H. pylori* infection, where the bacteria exclusively embed themselves under the acidic mucus gel layer of the gastric mucosa surface. This prevents direct local antibiotic treatment due to both the extreme acidic condition of the stomach and the inaccessible site of the mucosa embedded bacteria. Moreover, coupled with the regular voidance of stomach contents and the high level of antibiotic resistance of the bacteria, it has proved difficult to eradicate *H. pylori* infections *in vivo* despite the fact that these bacteria are susceptible to many antibiotics *in vitro*. An antibiotic delivery system that spreads out and adheres well along the mucosal surface while slowly and effectively releasing the drug molecules into the mucosal layer is a potentially promising (at least partial) solution (Bardonnet, Faivre, Pugh, Piffaretti, & Falson, 2006). However, since good *in vitro* results do not guarantee an effective *in vivo* treatment, then *in vivo* tests are required to evaluate the potential of any *H. pylori* treatment system. To the best of our knowledge, existing drug delivery systems for the *in vivo* treatment of *H. pylori* are quite limited, with only one report on a delivery system of submicron size, that

* Corresponding author at: Department of Chemistry, Faculty of Science, Chulalongkorn University, Phayathai Road, Bangkok 10330, Thailand. Tel.: +66 2 2187634; fax: +66 2 2541309.
E-mail address: psupason@chula.ac.th (S. Wanichwecharungruang).

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of ~300 nm sized nanoparticles made from the wheat glycoprotein gliadin with a 62% (w/w) amoxicillin loading that was used to treat *H. pylori* infected Mongolian gerbils (Umamaheshwari, Ramteke, & Jain, 2004). Other existing reports include the use of micron size carriers to treat *H. pylori* infected Mongolian gerbils, including the use of 85.7–129.7 μm floating ethyl cellulose (EC) spheres with less than 10% (w/w) clarithromycin (Rajinikanth, Karunagaran, Balasubramaniam, & Mishra, 2008), or 250–335 μm size spheres made from curdlan and carboxyvinyl polymer with ~4% (w/w) amoxicillin loading (Nagahara et al., 1998). Other clarithromycin encapsulation systems that have been evaluated *in vitro*, but with no *in vivo* tests, include cyclodextrin (Salem & Düzgünes, 2003), liposome (Bardonnet, Faivre, Boullanger, Piffaretti, & Falson, 2008; Salem, Flasher, & Düzgüneş, 2005), poly(lactic-co-glycolic acid) nanospheres (Mohammadi et al., 2011) and chitosan hydrochloride-genipin crosslinked microspheres (Harris, Lecumberri, & Heras, 2010). Thus, it would be interesting to see if nano-sized carriers made from inexpensive edible ethyl cellulose (EC), a modified natural carbohydrate, using a simple anti-solvent particle induction method, could truly enhance the effectiveness of clarithromycin in combating the *H. pylori*, both *in vitro* and *in vivo*.

Study of ethyl cellulose in the form of nanocarriers is still limited, with only one report on their mucoadhesion property (Suwannateep et al., 2011), therefore, it is interesting to see if this inexpensive polymeric carrier could help delivering drug to stomach mucosa for combating *H. pylori* infection. Here, we prepare clarithromycin-loaded EC nanoparticles and evaluate their (i) *in vitro* anti-*H. pylori* activity, using four different strains of the bacteria, (ii) *in vitro* anti-*H. pylori* adhesion activity, using Human laryngeal carcinoma (HEp-2) cells, and (iii) *in vivo H. pylori* clearance efficiency, using C57BL/6 mice infected with *H. pylori*. The simple yet effective anti-solvent particle induction method to prepare water dispersible clarithromycin-loaded nanoparticles from EC polymer was also demonstrated for the first time.

2. Materials and methods

EC (viscosity 250–300 cp; ethoxy content 48%) was purchased from Sigma–Aldrich (Steinheim, Germany). Acetone (analytical grade) was purchased from Merck (Darmstadt, Germany). RPMI-1640 (with 2.05 mM L-glutamine) media and fetal bovine serum (FBS) were purchased from Hyclone (UT, USA). Clarithromycin was obtained from Siam Pharmaceutical Co., Ltd. (Bangkok, Thailand).

2.1. Encapsulation of clarithromycin

EC (150 mg) and clarithromycin (50 mg) were dissolved in acetone (50 ml). Then, water (200 ml) was dropped into the solution (50 ml) at the rate of 2 ml/min, while the solution was stirred continuously. The obtained aqueous suspension of clarithromycin-loaded EC nanoparticles (5.0 ml, 3.0 mg/ml polymer, 1.0 mg/ml clarithromycin) was then subjected to vacuum suction to remove acetone and then freeze-dry to obtain the dry product.

Encapsulation efficiency (% EE) and loading capacity (% loading) were evaluated by subjecting at least 5 ml of the obtained particle suspension (before being freeze-dry) to centrifugally filtering through MWCO 100,000 membrane (Millipore Amicon Ultra-15) at 2350 × *g* for 20 min to remove all the free clarithromycin. Then the obtained solid on the filter was soaked in 5–10 ml ethanol for 3 h at room temperature. The obtained ethanolic extract was pre-concentrated by subjecting to partial removal of ethanol by N_2 stream until the final volume was 3.0 ml. The concentrated extract was then quantified for clarithromycin using UV/Vis spectrophotometer at 278 nm, relying on a calibration curve constructed using standard clarithromycin solutions (0, 62.50, 125.0, 250.0, 500.0, 1000 ppm). Clarithromycin in the liquid filtrate was determined by first subjecting the filtrate to pre-concentration by vacuum removal of the water and acetone, then the concentrated filtrate was analyzed spectrophotometrically. % EE and % loading were calculated using Eqs. (1) and (2) as follows:

$$\%\ \text{EE} = \frac{\text{weight of clarithromycin found in the filtered particles}}{\text{weight of clarithromycin initially used}} \times 100 \quad (1)$$

$$\%\ \text{Loading} = \frac{\text{weight of clarithromycin found in the filtered particles}}{\text{weight of the filtered particles}} \times 100 \quad (2)$$

As a control for the evaluation of the encapsulation levels, the free clarithromycin concentration in the filtrate (unencapsulated) was also evaluated (as above), where this free clarithromycin plus the encapsulated amounts should equal the original amount loaded.

The obtained aqueous suspension of EC nanoparticles with or without clarithromycin loading were subjected to scanning electron microscopy (SEM) on a JSM-6400 instrument (JEOL, Ltd., Japan), and dynamic light scattering (DLS) on Mastersizer S and Zetasizer nanoseries instruments (Mulvern Instruments, Worcestershire, UK). The obtained clarithromycin-loaded particles, unloaded particles and pure clarithromycin were subjected to differential scanning calorimetry (DSC) at 0–300 °C under nitrogen with a scanning rate of 10 °C min^{-1} using a Netzsch DSC 204 (Netzsch Group, Germany).

2.2. In vitro anti-H. pylori assay

2.2.1. Bacterial strains and culture condition

The reference strains of *H. pylori* (ATCC 43504 and ATCC 43526) was purchased from the American Type Culture Collection (Rockville, MD), whilst two clinical isolated strains (HP001 and HP002) were obtained from Division of Gastroenterology, Department of Medicine, Thammasat University Hospital, Bangkok, Thailand. Brain heart infusion agar with 5% (v/v) sheep blood was used for bacterial growth and *H. pylori* was incubated at 37 °C under a microaerophilic condition (85% (v/v) N_2, 5% (v/v) O_2 and 10% (v/v) CO_2) using a gas generating kit (AnaeroPack-MicroAero, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) for 3 days.

2.2.2. Determination of minimum inhibitory concentrations (MICs)

The minimum inhibitory concentration (MIC) of free clarithromycin and the encapsulated clarithromycin were evaluated by the agar dilution method according to the National Committee for Clinical Laboratory Standards guidelines recommendations, using Mueller-Hinton agar with 5% (v/v) sheep blood (MHAS). Concentrations of encapsulated clarithromycin, free clarithromycin and amoxicillin were added to the MHAS, prior to pouring and setting, over the range of 0.0075–0.24 μg/ml, while metronidazole was used as above but at 8–512 μg/ml, all as two-fold dilutions. Stock samples were prepared in the appropriate medium, *i.e.*, encapsulated clarithromycin was dispersed in Muller-Hinton broth (MHB) at 48 μg/ml, clarithromycin was dissolved in methanol at 4.8 mg/ml, amoxicillin was dissolved in 0.1 M phosphate buffer pH 6.0 at 4.8 mg/ml and metronidazole was dissolved in dimethysulfoxide (DMSO) at 128 mg/ml. Each stock was diluted to the desired

concentration with MHB. Three-day-old bacterial colonies of *H. pylori* were suspended in 0.85% (w/v) aqueous NaCl solution and adjusted to 10^7–10^8 colony forming units (CFU) of *H. pylori*/ml. Then, 3 μl of *H. pylori* suspension was dropped onto each plate and incubated for 3 days as previously described (Chaichanawongsaroj, Amonyingcharoen, Saifah, & Poovorawan, 2010). The MIC was determined from visual examinations as the lowest concentration of each sample in the plate with no bacterial growth.

2.3. In vitro anti-adhesion of H. pylori toward HEp-2 cells

2.3.1. Culture of HEp-2 cells

The HEp-2 (human laryngeal carcinoma) cells were obtained from the Faculty of Allied Health Sciences, Chulalongkorn University and were grown on tissue culture plastic ware in RPMI 1640 media with 2.05 mM L-glutamine and supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (Gibco BRL Laboratories, Grand Island, NY, USA) at 37 °C in 5% (v/v) CO_2 with 80% humidity.

2.3.2. Anti-adhesion assay

The method of Beil&Kilian was used with some modification (Beil & Kilian, 2007). The HEp-2 cells were seeded on twenty-four well plates at a density of 10^5 cells/well and incubated at 37 °C in 5% (v/v) CO_2 with 80% humidity overnight. *H. pylori* (ATCC 43504) cells were suspended in 1.0 ml RPMI 1640 supplemented with 2.05 mM L-glutamine media (OD at 600 nm = 2.0) and then incubated with 10 μl of 0.1% (w/v) fluorescein isothiocyanate (FITC) in DMSO in the presence of the test sample (encapsulated or free clarithromycin, each at 0.12–0.48 μg clarithromycin/ml) for 2 h at room temperature in the dark. The mixture was then washed three times with RPMI 1640 media containing 0.1% (v/v) Tween 20, followed by washing twice with RPMI 1640 media to obtained FITC-labeled *H. pylori*. The HEp-2 cells were then infected with the FITC-labeled *H. pylori* (500 μl/well) at 37 °C in 5% (v/v) CO_2 with 80% humidity for 2 h, the media was withdrawn and the cells were washed with RPMI 1640 media three times. Finally, the amount of FITC-labeled *H. pylori* on the cells was determined by fluorescence spectrophotometry (excitation at 485 nm and emission at 528 nm). Differences in adhesion percentages were analyzed by one way analysis of variance (ANOVA) with Duncan's test. Statistical significance was accepted at the $p \leq 0.05$ level.

2.4. Preliminary in vivo eradication of H. pylori infection in mice

2.4.1. Sample preparation

The three samples, clarithromycin-loaded EC particles, empty EC-particles and free clarithromycin, were each prepared as a suspension in sterile water at a clarithromycin concentration of 1.8 mg/ml, or the equivalent amount of EC as in the encapsulated clarithromycin (5.4 mg/ml) for the empty EC-particles.

2.4.2. In vivo test

Sixteen C57BL/6 mice and the ATCC 43504 *H. pylori* strain were used. The procedures were approved by the ethics committee and the Institutional Animal Care and Use Committee (IACUC) of Chulalongkorn University (no. 11310079). Each mouse was repeatedly infected with 10^9 CFU of *H. pylori* after fasting for 8 h for three consecutive days. After 3 weeks, sixteen *H. pylori* infected mice of both sexes were randomly divided into four groups of three mice each. The animal groups were fed once a day *per os* using a feeding needle for three consecutive days with one of (i) distilled water (positive control group and uninfected control group), (ii) unencapsulated clarithromycin, (iii) clarithromycin-loaded EC particle suspension, (iv) unloaded EC particle suspension, at a clarithromycin dose of 30 mg/kg bodyweight (or for the unloaded EC particles at the equivalent amount of EC as the clarithromycin-loaded EC particles). Forty eight hours after administration of the final dose, the mice were sacrificed and the stomach samples were collected and fixed in 10% (v/v) neutral buffered formalin. The samples were histologically processed and embedded in paraffin prior to sections being cut with an ultramicrotome at 4 μm thickness and stained with hematoxylin and eosin (H&E) and Warthin-Starry method. The immuno-histochemical staining was performed using a specific *H. pylori* antibody. Briefly, the 4-μm-thick tissue sections were pretreated by 0.1% (w/v) trypsin for 25 min at 37 °C, and then treated with 3% (v/v) H_2O_2 for 15 min at room temperature to inactivate endogenous peroxidase and then blocked with 1% (w/v) bovine serum albumin (BSA) in phosphate buffer saline (PBS) pH 7.4 for 45 min at 37 °C. The tissue samples were incubated with polyclonal rabbit anti-*H. pylori* antibody (ready-to-use-kit, Dako, Glotrup, Denmark) overnight at 4 °C, washed 3 times with PBS, and then incubated with a chain polymer kit (DakoEnVision$^+$ System-HRP, anti-rabbit, Dako, Tokyo, Japan) for 45 min at 37 °C. Finally, the presence of antigens was verified visually with a light microscope after the samples were treated with 3,3′-diaminobenzidine-4HCl (DAB) coupled with hematoxylin counterstaining. The semi-quantitative evaluation was blindly observed and the immunoreactivity of each sample was graded according to the Sydney system as negative, low, moderate and numerous.

3. Results and discussion

3.1. Encapsulation

Clarithromycin was successfully encapsulated into EC nanoparticles at a 3:1 weight ratio of EC: clarithromycin by a simple yet effective anti-solvent particle induction method in which water was slowly added to the solution of EC and clarithromycin in acetone. Upon water (anti-solvent) addition, the clear solution of clarithromycin and EC in acetone (good solvent) turned cloudy. With the slow change of medium from acetone (solvent) rich to water (anti-solvent) rich, it is likely that the largely hydrophobic clarithromycin molecules would precipitate or form into small crystals and the EC chains slowly self-assemble themselves around the crystals. The EC chains probably orientated the hydrophilic moieties (hydroxyl groups) in contact with water richer medium while the hydrophobic moieties (ethoxyl and methylene groups) would fold inside in contact with the clarithromycin molecules and so form particles in order to have minimal contact with the water. The formed particles, therefore, contained hydrophilic hydroxyl groups at the outer surface of the particles and the drug crystals at the inside of the particles. This resulted in the water dispersible particles, even though the bulk EC is water insoluble and the system contained no surfactant.

SEM analysis of the particles revealed that they were mainly broadly semi-spherical and largely individual particles, or at least not strongly agglomerated and with a diameter of 223 ± 50 nm for the dry particles (Fig. 1), whilst DLS indicated hydrodynamic diameters of 449 ± 12 nm with a PDI of 0.403 ± 0.034 (mid-range polydispersity).

To find the loading of clarithromycin in the particles and encapsulation efficiency of this anti-solvent particle induction process, the obtained particle suspension were centrifugally filtered through Amicon Ultra-15 MWCO 100,000 membrane. Free (unencapsulated) clarithromycin molecules passed through the membrane while those inside the particles were retained on the filter. Extraction, pre-concentration and quantification of the filtered spheres revealed that 86 ± 0.5% of the initial clarithromycin level added to the mixture was present (encapsulated), in agreement

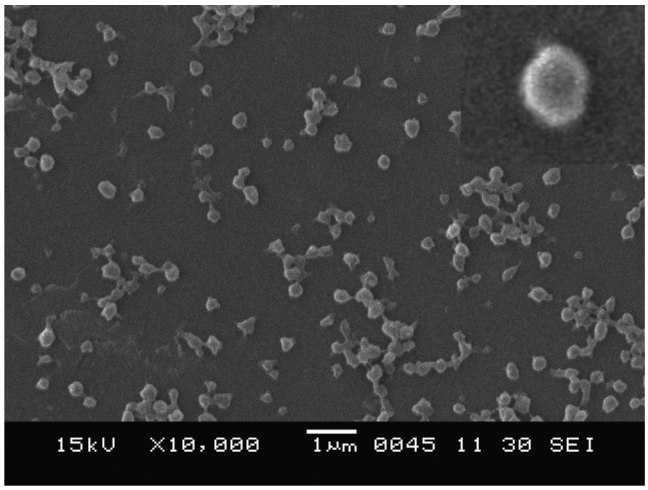

Fig. 1. SEM images of the clarithromycin-loaded EC particles. Inset shows the expansion of the particle image.

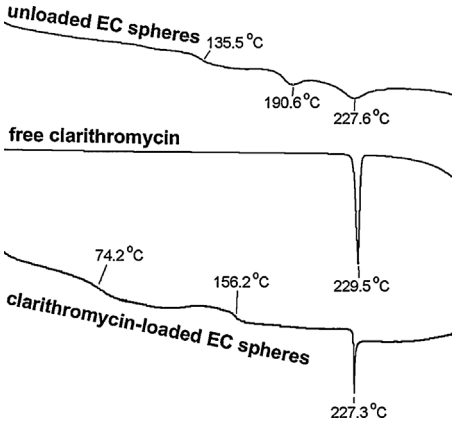

Fig. 2. Thermograms of the unloaded EC particles, free clarithromycin and clarithromycin-loaded EC particles.

with the corresponding ~13% of residual (free or unencapsulated) clarithromycin found in the liquid filtrate (quantified after being pre-concentrated). Therefore, the EE of the process was 86 ± 0.5% and the clarithromycin loading of the particles was 22.3 ± 0.17% by weight. It should be noted here that linear calibration curve of the standard clarithromycin could be obtained with the r^2 value of 0.9997, indicating that the UV–vis spectrophotometric method could still be reliably used for clarithromycin quantification in the concentration ranges used in the experiment, regardless of the compound low molar absorptivity. Not only the method gave repeatable results but also the accuracy was proof through the fact that the amount of encapsulated clarithromycin plus the amount of clarithromycin found in the liquid filtrate equaled the original amount of clarithromycin used in the particle fabrication process.

The DSC analysis revealed a glass transition temperature (T_g) at 74.2 °C and 156 °C for the clarithromycin-loaded EC particles, while that for the unloaded EC particles showed a T_g at 136 °C (Fig. 2). Two broad absorption peaks at 190.6 °C and 227.6 °C were observed for the unloaded EC particles while the loaded particles showed only one sharp absorption peak at 227.3 °C. The melting temperature of pure clarithromycin was 229.5 °C. The change in the T_g and absorption temperature upon clarithromycin encapsulation indicated a good interaction between the EC polymer chains and the clarithromycin molecules. Although the clarithromycin-loaded EC particles, pure clarithromycin and the empty EC particles all showed an absorption peak at 227–229 °C, this was much sharper in the thermogram of the clarithromycin-loaded EC particles compared to that of the unloaded EC particles, and so indicates the likely presence of crystalline clarithromycin in the encapsulated system. Thus, it was speculated that clarithromycin molecules encapsulated in the EC particles were in both a solid solution and crystalline states. We speculate that during the slow water addition, clarithromycin crystals formed probably act as seed for the deposition point of the polymers during the above described self-organization of the drug-polymer nanospheres. It should be noted here that no unencapsulated clarithromycin crystals/precipitate were observed in the product when carefully analyzed by SEM (Fig. 1). In fact, result from our preliminary experiment performed at different drug to polymer ratios (1:1, 1:2 and 1:3) has confirmed that at the drug to polymer ratio of 1:3 and under the condition stated in the experimental section, no free clarithromycin crystal was detected under the microscope (see supplementary data). Obvious clarithromycin crystals could be observed under microscope when encapsulation was performed at drug to polymer ratio of 1:1.

The clarithromycin-loaded EC particles dispersed well in both simulated gastric fluid (SGF), prepared according to the official compendia of standard USP 28, NF 23, United States Pharmacopeia and National Formulary, and water. No precipitation was observed when the particle suspension was mixed with SGF. This well dispersed characteristic is an important requirement for the spheres to be able to spread evenly and efficiently along the stomach mucosa during *in vivo* administration and thus is a basic requirement for any delivery system to be used for the treatment of *H. pylori* infections in the stomach.

3.2. In vitro anti-H. pylori activity

The encapsulated clarithromycin was tested for its *in vitro* anti-*H. pylori* activity using four different strains of *H. pylori* and the results, shown as the minimal inhibition concentration (MIC) of the tested materials, revealed a two- to four-fold lower activity (0.09–0.18 μg/ml) compared to that for the free clarithromycin (0.03 μg/ml) (Table 1). This agrees well with the notion that the polymeric EC shell barrier would hinder instant release and so instant action of the encapsulated clarithromycin on the *H. pylori* bacteria compared to free (unencapsulated) clarithromycin. However, note also that DMSO was used to aid the poor water solubility of free clarithromycin in the assay while clarithromycin-loaded particles were used as water suspension with no organic solvent. Therefore, the improvement in water dispersibility by encapsulation could not be observed by this test. The test, however, confirmed

Table 1
Anti-*H. pylori* activity.

Stains	MIC (μg/ml)			
	Metronidazole	Amoxicillin	Clarithromycin	Encapsulated clarithromycin
ATCC 43504	64	0.03	0.03	0.09
HP001	32	0.015	0.03	0.09
HP002	32	0.03	0.03	0.09
ATCC 43526	≤32	0.06	0.03	0.18

that the drug was still active after being encapsulated. Compared to the activity noted with metronidazole and amoxicillin, two commonly used drugs for the treatment of *H. pylori* infections, the encapsulated clarithromycin revealed an acceptable activity level.

3.3. Anti-H. pylori adhesion activity

The ability to prevent *H. pylori* bacteria from adhering to the target surfaces of the host's organ (stomach) is one of the important mechanisms required for combating *H. pylori* infection during *in vivo* treatment. Since the previous report indicated the similarity between cells of the HEp-2 cell line and porcine gastric epithelial cells (Kobayashi, Okazaki, & Murakami, 1993), clarithromycin-loaded particles were tested for anti-adhesion activity *in vitro* on HEp-2 cells. Accordingly, it is assumed to be likely that the ability to prevent *H. pylori* bacteria from adhering to the HEp-2 cells *in vitro* may also represent the ability of the material to prevent these bacteria from adhering to the gastric epithelial cells (*in vivo*). Compared to the control, free clarithromycin only partially inhibited *H. pylori* binding to the HEp-2 cells and only at the highest tested dose of 0.48 mg/ml (Fig. 3). In contrast, the encapsulated clarithromycin showed a strong and dose-dependent inhibition of *H. pylori* adhesion. This enhanced activity was probably a result of the good adherence of the EC particles on the surface of the HEp-2 cells, presumably due to hydrogen bonding between the hydroxyl group at the surface of the EC spheres and the sugar moieties at the surface of the HEp-2 cells. Although DMSO was used to aid dissolution of the unencapsulated clarithromycin in the assay, the free drug still showed lower ability to prevent adhesion of the bacteria toward HEp-2 cells when compared with the encapsulated clarithromycin. DMSO (at concentration used in the assay) and unloaded EC particles showed no significant anti-adhesion activity (Fig. 3). The enhanced anti-adhesion activity of the encapsulated clarithromycin is encouraging for its potential use in the *in vivo* treatment and eradication of *H. pylori* infections.

3.4. In vivo eradication of H. pylori in mice

Four treated groups of the *H. pylori* infected mice, (i) control, (ii) unencapsulated EC particle treated, (iii) free clarithromycin treated and (iv) the encapsulated clarithromycin treated, were immunohistochemically evaluated for the presence of *H. pylori* after treatment (Table 2). A lot of short curved and coccoid forms of *H. pylori* were observed in the fundus and pylorus of the untreated (positive control) group and the unloaded EC particle-treated group. Thus, the EC particles themselves appeared to cause no significant reduction in *H. pylori* infection levels. The numbers of positive immunostaining bacteria were significantly reduced in the free clarithromycin and especially in the encapsulated clarithromycin treatment groups.

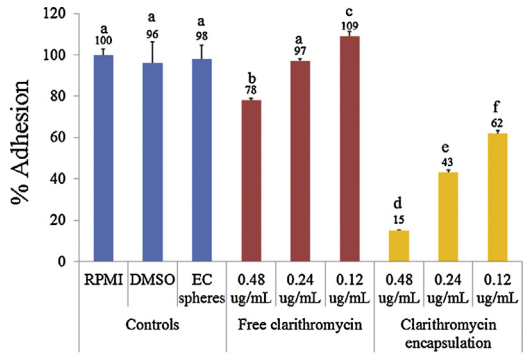

Fig. 3. Ability of encapsulated clarithromycin to inhibit adhesion of *H. pylori* to the surface of HEp-2 cells. (Red) Free clarithromycin (dissolved in water with DMSO to aid the solubilization), (yellow) encapsulated clarithromycin (as water suspension with no organic solvent) and (blue) the controls of medium alone (RPMI), medium with DMSO at the same concentration to those used in the free clarithromycin treated groups, or unloaded EC particles. Data are shown as the mean ± 1 SD, derived from 3 independent repeats. Means with a different lower case letter are significantly different ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Immunohistochemical staining results for the detection of *H. pylori*.

Group	Grading of positive staining ($n = 3$)
(1) *H. pylori* positive control	+++
(2) Unloaded EC particle	+++
(3) Free clarithromycin	Between ++ and +++
(4) Encapsulated clarithromycin	Between + and ++

Immunoreactivity scale: − = negative; + = low; ++ = moderate; +++ = numerous.

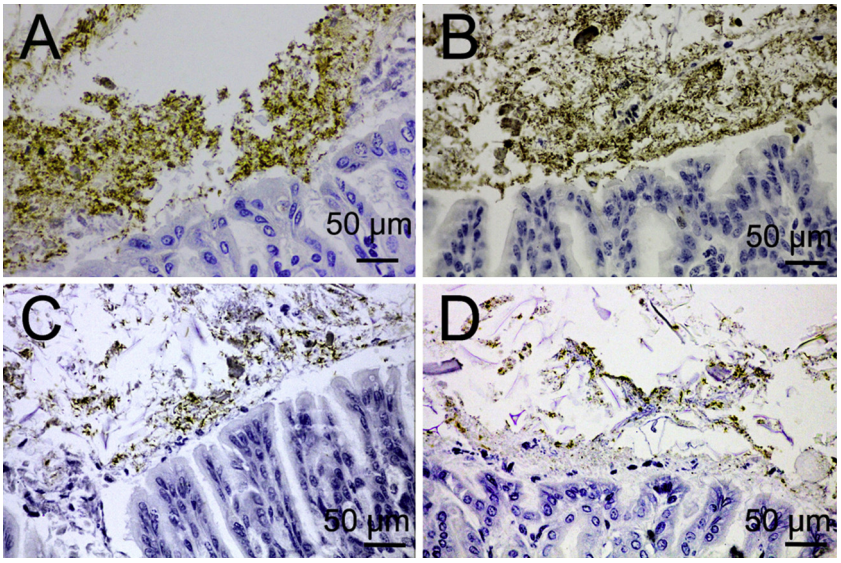

Fig. 4. Immunohistochemical demonstration of *H. pylori* in the stomach. (A and B) High numbers of short curved bacilli and coccoid *H. pylori*-positive immunostaining cells in the stomach content of the (A) *H. pylori* positive control and the (B) unloaded EC particle-treated groups. In contrast, (C and D) a low number of positive stained bacteria were observed in the (C) free Clarithromycin treated and the (D) encapsulated clarithromycin-treated groups. In (D) note not only the lowest density of immunostained bacteria but also the crumbled bacterial morphology. Envision+ system-HRP, DAB method.

As expected, the stomach of the encapsulated clarithromycin-treated mice showed significantly less *H. pylori* than those of the free clarithromycin-treated mice. In addition, the positive stained bacteria observed in the encapsulated clarithromycin-treated group appeared more crumbled compared to the other groups (Fig. 4A–D). The immunostaining of bacteria in Fig. 4D shows that the bacteria was not in the coccoid or bacilli morphology and they could not adhere on the mucosa. The reason for the better activity of the encapsulated clarithromycin compared to the free drug is likely to be the result of the good adherence of clarithromycin-loaded EC nanoparticles along the stomach mucosa of the treated mice (Suwannateep et al., 2011). Adhesion of the drug loaded particles at the mucosa directly helps localize the drug at the habitat of *H. pylori* and allows a local drug (clarithromycin) concentration gradient to form. This preliminary result indicates a good potential of the clarithromycin-loaded EC nanoparticles for the orally administered treatment of *H. pylori* infections. This result agrees well with previous report on stomach mucosa attachment of the self-assembled nanoparticles made from EC (Suwannateep et al., 2011).

4. Conclusion

Here we showed that nanoparticles made from EC through a simple anti-solvent particle induction method could effectively be loaded with clarithromycin. The obtained submicron sized semi-spherical particles (223 ± 50 nm anhydrous diameter and 449 ± 12 nm hydrodynamic diameter) possessed $22.3 \pm 0.17\%$ (w/w) clarithromycin loading at a $86 \pm 0.5\%$ encapsulation efficiency. The particles dispersed well in water and SGF and gave a MIC of 0.09–0.18 μg/ml against four strains of *H. pylori*. Encapsulation of clarithromycin into EC nanoparticles also enhanced the anti-adhesion activity of clarithromycin when tested with *H. pylori* and HEp-2 cells. Preliminary *in vivo* trials in *H. pylori* infected mice clearly indicated better eradication of the bacteria from the stomach by the clarithromycin-loaded EC nanoparticles compared to the free drug.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.03.025.

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