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Stomach-specific anti-*H. pylori* therapy Part III: Effect of chitosan microspheres crosslinking on the gastric residence and local tetracycline concentrations in fasted gerbils

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Abstract

The main objective of the present study was to examine the effect of chemical crosslinking of chitosan microspheres on the gastric residence and local tetracycline concentrations following oral administration in fasted gerbils. Radioiodinated [^{125}I] glyoxal-crosslinked chitosan microsphere suspension in deionized distilled water was administered for the gastric residence studies. At different time points, the animals were sacrificed and the radioactivity in tissues and fluids was measured. Stomach tetracycline concentrations were determined using tritiated-[^{3}H]-tetracycline-loaded crosslinked chitosan microspheres. The radioactivity, measured with a liquid scintillation analyzer, was used to determine the microgram of drug per gram of tissues or fluids. After 2 h in the fasted stomach, approximately 10% of the non-crosslinked chitosan microspheres remained. On the other hand, 17% of the crosslinked chitosan microspheres remained in the fasted stomach after the same time period. The microspheres were predominantly found in the colon after 6 h of administration. There was no detectable radioactivity in the plasma, urine, small intestine, liver, and kidneys. Tetracycline concentration profile in the stomach from the crosslinked microsphere formulation was higher than that of the aqueous solution and the non-crosslinked microsphere formulation. While the area-under-the-curve ($AUC_{0.5\rightarrow 10\,h}$) for tetracycline solution and non-crosslinked chitosan microspheres was 447.3 and 358.2 μg h/g of tissue, respectively, the $AUC_{0.5\rightarrow 10\,h}$ for the crosslinked chitosan microspheres was 868.9 μg h/g of tissue. The drug was predominantly found in the colon and urine after 6 h of administration. Results of this study show that chitosan microspheres prepared by chemical crosslinking provide a longer residence time in the fasted gerbil stomach than either tetracycline solution or microspheres prepared by ionic precipitation.

Keywords: Stomach-specific delivery; Chitosan microspheres; Chemical crosslinking; Glyoxal; Tetracycline; *Helicobacter pylori*; Gastric residence time

1. Introduction

The etiologic link between *Helicobacter pylori* infection and chronic gastritis, peptic ulcer disease, and gastric cancer has been clearly established since the organisms were first isolated 23 years ago by Warren and Marshall (Marshall, 1983; Blaser, 1990). Although a number of antibacterial agents, such as amoxicillin and tetracycline, have very low minimum inhibitory concentration (MIC) values against *H. pylori* in culture, single antibiotic therapy is not effective in the eradication of *H. pylori* infection in vivo. This is because of the low concentration of the antibiotic

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reaching the bacteria under the mucosa, instability of the drug in the low pH of gastric fluid, and the short residence time of the antibiotic in the stomach (Shah et al., 1999). Combination of more than one antibiotic and anti-secretory agent, therefore, is required for the complete eradication of *H. pylori*. Patient compliance for the combination therapy is very low mainly due to the side effects, cost of therapy, and the lack of willingness to take many different drug products. *H. pylori* resistance to single and multiple antibiotic therapy is also on the rise in the United States and other parts of the world (Megraud, 1994; Reddy et al., 1996; Vasquez et al., 1996). In order to reduce the level of resistance, several investigators have proposed the use of non-antibiotic anti-*H. pylori* agents and the development of therapeutic strategies for complete eradication of *H. pylori* from the stomach.

In order to improve the efficacy of anti-*H. pylori* agents, we and others have proposed that local delivery in the stomach may also enhance the therapeutic benefits (Akiyama et al., 1995; Nagahara et al., 1998; Hejazi and Amiji, 2002). Using acid-stable antibiotics like amoxicillin, metronidazole, and tetracycline (Shah et al., 1999; Hejazi and Amiji, 2002), stomach-specific delivery system will increase the gastric residence time, decrease the diffusional distance, and allow more of the antibiotic to penetrate through the gastric mucus layer and act locally at the infectious site. By increasing the local concentration and contact time, stomach-specific delivery system may also minimize the resistance problems associated with systemic administration of antibiotics.

In a previous study (Hejazi and Amiji, 2002), we described the development of tetracycline-loaded chitosan microspheres for stomach-specific delivery. Chitosan [$\alpha(1 \rightarrow 4)$ 2-amino-2-deoxy-β-D-glucan] is obtained by the alkaline deacetylation of chitin. Chitin, an abundant biopolymer, is mainly harvested from the exoskeleton of crustaceans like shrimp, lobster, krill, and crab (Roberts, 1992). In the cationic form, the D-glucosamine residue of chitosan interacts with the *N*-acetylneuraminic acid (sialic acid) residues of mucin by electrostatic forces (Lehr et al., 1992). The muco/bioadhesive properties of chitosan may allow a prolonged interaction of the delivered drug with the membrane epithelia facilitating more efficient drug diffusion into the mucus/epithelial cell layer. Also, chitosan has intrinsic anti-ulcer effect by adhesive action on gastric epithelial cells and/or by neutralizing the hydronium ions in the gastric fluid (Ito et al., 2000). The microspheres, prepared by ionic precipitation, were spherical in shape and had an average diameter of 2.0–3.0 μm. Fifty-five percent of tetracycline was loaded into the chitosan microspheres, and the drug was stable for up to 12 h even under highly acidic condition. In acidic medium (pH 1.2–2.0), the microspheres dissolved and the entrapped drug was released instantaneously. However, 60% of the entrapped drug was released in 2 h when the pH of the medium was raised to 3.5.

In a follow up study (Hejazi and Amiji, 2003), we examined the gastric residence time of chitosan microspheres following oral administration in fasted gerbils. Because of the instant dissolution of the chitosan microspheres in acidic medium of up to pH 2.0, we also examined the effect of acid suppression on the chitosan microspheres residence time and the tetracycline concentrations in the stomach. We postulated that the higher pH of the stomach would allow the microspheres to remain intact and thus, prolong the residence time. Results of that study showed that chitosan microspheres prepared by ionic precipitation did not provide a longer residence time in the fasted gerbil stomach. The tetracycline concentration profile in the stomach, following administration in microsphere formulation, was similar to that of aqueous drug solution. Also, acid suppression did not influence the gastric residence time of chitosan microspheres or tetracycline concentration profiles.

Since the chitosan microspheres prepared by ionic precipitation were formed by the neutralization of the positively charged amino groups with the sulfate ions, the high proton concentration in acidic media will shift the equilibrium towards solubilization of the microspheres. In addition, we observed that the microspheres prepared by ionic precipitation did not settle in the stomach. Chemical crosslinking is a way of overcoming the problem of instant dissolution and allow the particles to be harder and settle in the stomach for effective muco/bioadhesion. Thus, the purpose of this study was to prepare glyoxal-crosslinked chitosan microspheres that resisted dissolution and to investigate the gastric residence of the microspheres and local tetracycline concentrations following oral administration in gerbils.

2. Materials and methods

2.1. Materials

Chitosan with an average viscosity molecular weight of 750,000 Da and with 87% degree of deacetylation was obtained from Pronova Biopolymers (Raymond, WA). Tetracycline HCl was purchased from ICN (Aurora, OH). Tritiated [^{3}H]-tetracycline and radioiodine [^{125}I]-labeled Bolton–Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-3-[^{125}I]iodophenyl)propionate) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Glacial acetic acid, hydrochloric acid, hydrogen peroxide, sodium chloride, and sodium sulfate were purchased from Fisher Scientific (Pittsburgh, PA). Tween 80 was purchased from City Chemical Corporation (Jersey City, NJ). Glyoxal and glycine were purchased from Sigma (St. Louis, MO). All aqueous solutions were prepared using deionized distilled water (Nanopure II, Barnstead/Thermolyne, Dubuque, IO). The other reagents and chemicals were of analytical grade or better.

2.2. Preparation of tetracycline-loaded chitosan microspheres

Chitosan microspheres were prepared by ionic precipitation and chemical crosslinking (Berthold et al., 1996). Briefly, sodium sulfate (20%, w/v) was added drop-wise to a stirring solution of chitosan (0.25%, w/v in 0.1 M acetic acid) and Tween 80 (1%, w/v), until uniform turbidity was detected. The suspension was further stirred for an additional hour to stabilize the microspheres. The microsphere suspension was centrifuged at 2000 × *g* for 30 min and the pellet was washed twice with deionized distilled water. Glyoxal was added to the microsphere suspension in deionized distilled water at a final concentration of 0.5% (w/v) and allowed to crosslink for 1 h at 25 °C. The final concentration of glyoxal was optimized to insure that the formed microspheres did not dissolve in acidic media (pH 1.2–2.0). The crosslinked microsphere suspension was centrifuged at 2000 × *g* for 30 min and the pellet was washed with 0.1-M glycine solution to mask the unreacted aldehyde groups. Since glyoxal is a well-known mucosal irritant, the microspheres were washed twice using deionized distilled water to remove the unreacted glyoxal. The microspheres were isolated, frozen in liquid nitrogen, and lyophilized. Tetracycline solution (50 mg/ml) was incubated with the crosslinked chitosan microspheres (1:1 weight ratio) for up to 48 h in a shaking (55 rpm) water-bath. After separating the bound drug from the free, we observed that 85% of tetracycline was loaded into the crosslinked chitosan microspheres.

2.3. Characterization of chitosan microspheres

2.3.1. Particle size analysis

To insure reproducibility from batch-to-batch, the suspension, formed after glyoxal crosslinking and before lyophilization, from each microsphere sample was used for particle size analysis using Beckman/Coulter N4® plus (Fullerton, CA) instrument. The size of the microspheres was measured at a 90° scattering angle and at 25 °C.

2.3.2. Differential interference contrast (DIC) microscopy

A few drops of chitosan microsphere suspension, formed after glyoxal crosslinking and before lyophilization, were placed on a clean glass slide. After placing a cover-slip on the sample, it was observed with Zeiss Axioplan-2® confocal microscope (Thornwood, NY). DIC image of the microsphere sample was digitized and processed with Adobe Photoshop® software.

2.3.3. Transmission electron microscopy (TEM)

A few drops of the resuspended freeze-dried chitosan microspheres were added on a formvar coated grid. The size and the shape of the microspheres were observed using JEOL transmission electron microscope JEM1010 (Peabody, MA) at an accelerating voltage of 60.0 kV. The TEM image was digitized and processed with Adobe Photoshop® software.

2.3.4. Surface charge measurements

The surface charge on unloaded and tetracycline-loaded chitosan microspheres was measured from zeta potential values in deionized distilled water with a Brookhaven Instrument's ZetaPALS® (Phase Analysis Light Scattering) Ultra-Sensitive Zeta Potential Analyzer (Holtsville, NY).

2.4. In vitro drug release studies

Drug-loaded chitosan microspheres (3 mg) were incubated with 1.5 ml of hydrochloric acid solutions adjusted to pH 1.2 and 2.0 and 0.1 M acetate buffers adjusted to 3.5 and 5.0 in an Eppendorf vial in a shaking water-bath at 37 °C. The ionic strength of the release medium was adjusted to 0.16 using sodium chloride. At each time point, the vial was centrifuged at 10,000 × *g* for 5 min and 1 ml of supernatant was withdrawn. To maintain sink condition, 1 ml of fresh release medium was added to the vial. The sample was diluted 20 times and assayed for the released tetracycline at 350 nm using Shimadzu UV160U spectrophotometer. The cumulative amount of tetracycline was obtained from the calibration curves of tetracycline in each of the release medium.

2.5. Animal model

All of the animal experiments described here were approved by the Northeastern University's Institutional Animal Care and Use Committee (protocol: 010101R). Male Mongolian gerbils weighing between 41 and 50 g (6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). The supplier had confirmed to us that these gerbils did not harbor any gastric pathogens that may cause infection or interfere with the analysis. After shipment, the gerbils were divided into groups of four animals and kept in separate cages with 12 h of light and dark cycles. Food and water were supplied ad libitum. Prior to any experiment, the gerbils were allowed to acclimate to their new environment for 48 h.

2.6. Gastric residence time of chitosan microspheres

2.6.1. Radioiodination of chitosan microspheres

^{125}I-Labeled Bolton–Hunter reagent, with a specific activity 2200 Ci/mmol, was supplied in anhydrous benzene in a specially washed and dried NENSURE® vial (Perkin-Elmer Life Sciences, Boston, MA). Bolton–Hunter reagent is specifically used for labeling amine groups in proteins. According to the supplier's instruction, optimum labeling efficiency is obtained when the reaction is performed in the NENSURE® vial. After inserting a charcoal trap into the septum of the NENSURE® vial, benzene was evaporated to dryness by a gentle stream of nitrogen gas. Chitosan microspheres (17 mg) were wetted with 400 μl of borate buffer (pH 8.5) and added to the vial that contained the dry iodinated ester. The sample was agitated for 15 min at room temperature. To remove unbound ^{125}I-Bolton–Hunter reagent, the mixture was centrifuged at 5000 × *g* for 5.0 min, washed with borate buffer, and a small aliquot was removed for radioactivity measurement. The washing steps were repeated until all of the unbound ^{125}I-Bolton–Hunter reagent had been washed out. This was confirmed when the supernatant radioactivity counts had reached a minimal value. Finally, the microspheres were air-dried for 24 h in a well-ventilated hood. In our previous study (Hejazi and Amiji, 2003), it was demonstrated that the amide bond formed by the reaction of ^{125}I-labeled Bolton–Hunter reagent with the amine groups of chitosan was stable in the acidic environment of the gastric fluid.

2.6.2. Dosing protocol

A total of 24 animals needed for gastric retention study were fasted for 24 h. These studies were performed under fasted conditions since the peristaltic contraction force is more pronounced in this state (Phase III or "house-keeper wave") than in the fed state. The ^{125}I-labeled chitosan microspheres (specific activity: 0.075 μCi per 1 mg of chitosan microspheres) were suspended in deionized distilled water before oral administration. Using a feeding needle (length 38.1 mm, ball diameter 2.25 mm) and syringe, each animal was dosed 3.0 mg of radiolabeled chitosan microspheres suspended in 0.3 ml of water.

2.6.3. Measurement of microsphere concentrations in the stomach

At 0.5, 1, 2, 4, 6, and 10 h post-administration, a group of four gerbils for each time point were sacrificed by CO_2 inhalation. The stomach was excised, washed by phosphate buffer solution, and blotted on a piece of Kimwipe® tissue (Kimberly-Clark, Roswell, GA) to remove excess fluid. After recording the weight, the whole stomach was placed in a test tube and the radioactivity was measured with a Perkin-Elmer Wallac WIZARD 1470 automatic gamma counter (Boston, MA). The phosphate buffer saline rinse was also assayed for any radioactivity. The measured radioactivity in counts-per-minute was

converted into specific activity (μCi) and percent of dose administered using appropriate standards.

2.6.4. Measurement of microsphere concentrations in different tissues

Besides the stomach concentration, we were interested in investigating the transit time of the microspheres across the gastrointestinal tract. At 6 h post-administration to animals, stomach, small intestine, colon, kidney, and liver were excised and their weights were recorded. All of the tissues were processed and the radioactivity was measured as described above. To determine the concentration of microspheres in plasma and urine, the animals were housed in metabolic cages after dosing. Following euthanasia, blood was withdrawn by cardiac puncture in heparinized tubes (Vacutainers®, Becton-Dickinson, Franklin Lakes, NJ). Plasma was obtained from whole blood by centrifuging at 2500 × *g* for 10 min. After recording the volumes of plasma and urine samples, radioactivity was measured as described earlier. Since animals were fasted for 24 h prior to dosing, fecal matter could not be collected.

2.7. Measurement of local tetracycline concentrations

2.7.1. Preparation of [^{3}H]-tetracycline-containing chitosan microspheres

Stock solution of tetracycline was prepared by proportional mixing of 0.012 μCi labeled tetracycline to 1.0 mg of cold tetracycline in deionized distilled water. Chitosan microspheres, prepared as described earlier, were incubated with tetracycline solution (50 mg/ml) in a 1:1 weight ratio for 48 h in a shaking (55 rpm) water-bath. The microsphere suspension was centrifuged at 2000 × *g* for 30 min and the supernatant was discarded. The drug-loaded microspheres were rapidly frozen in liquid nitrogen and lyophilized. With a loading capacity of 85% (w/w), a 100 mg sample of lyophilized chitosan microspheres had 85 mg tetracycline and an activity of 1.02 μCi. The lyophilized microspheres were suspended in deionized distilled water such that the concentration of tetracycline in the final mixture was 5.0 mg/ml. A mixture of hot and cold tetracycline solution in deionized distilled water was also prepared at the same concentration and used as a control.

2.7.2. Dosing protocol

Forty-eight gerbils were divided into control and test groups of four animals each. Animals were fasted for 24 h before administration of the tetracycline solution (control) and the tetracycline-chitosan microsphere formulation (test). Using feeding needle, each animal was dosed orally with 0.3 ml of the microsphere suspension containing 1.5 mg of the drug.

2.7.3. Analysis of drug in the stomach

At 0.5, 1, 2, 4, 6, and 10 h post-administration, the gerbils were sacrificed by CO_2 inhalation. The stomach tissue was surgically excised, an incision was made through the greater curvature, and it was washed with phosphate buffer (pH 7.4). After recording the weight, the stomach was homogenized in deionized distilled water using PowerGen® 125 tissue homogenizer (Fisher Scientific, Pittsburgh, PA) to make a 10% (w/v) homogenate concentrate. In each scintillation vial, 1.0 ml of Scintigest® (Fisher Scientific, Pittsburgh, PA), a tissue solubilizer, was added to 1.5 ml of the homogenate and the mixture was incubated at 55 °C for 2 h. Upon cooling to room temperature, samples were decolorized with 200 μl of hydrogen peroxide (30%, w/w). Following a re-incubation at 55 °C for 30 min, 10 ml of ScintiSafe Econo 1® (Fisher Scientific, Pittsburgh, PA), the liquid scintillation cocktail, was added to each vial. The radioactivity in the stomach homogenate was measured with a Packard Instrument's Tri-Carb 1600 TR liquid scintillation analyzer (Meriden, CT). The phosphate buffer saline rinse was also assayed for radioactivity. The measured radioactivity, as count per minute (CPM), was converted to microgram of drug using appropriate standards.

2.7.4. Measurement of tetracycline concentration in tissues and fluids

In order to determine the concentration of tetracycline in plasma, urine and tissues at 6-h post-administration of the control and test formulations, the animals were housed in metabolic cages after dosing. Following euthanasia, blood was withdrawn by cardiac puncture in heparinized tubes (Vacutainers®, Becton-Dickinson, Franklin Lakes, NJ). In addition, stomach, small intestine, colon, kidney, and liver were excised and their weights were recorded. Again due to 24-h fasting, fecal matter could not be collected from

animals. Plasma was obtained from whole blood by centrifuging at 2500 × *g* for 10 min. After recording the volumes of plasma and urine samples, radioactivity was measured as described earlier. Tissue samples were homogenized in deionized distilled water and the radioactivity was measured after tissue solubilization and decolorization steps as described above.

2.8. Data analysis

Statistical analysis of the results was performed with Microsoft Excel® (Microsoft Corporation, Redmond, WA). Mean and standard error of the mean (S.E.M.) were calculated from at least four independent experiments. Statistical significance was determined at 95% confidence interval ($P \leq 0.05$).

3. Results and discussion

3.1. Characterization of microspheres

Coulter analysis of the suspension, formed after glyoxal crosslinking and before lyophilization, showed that crosslinked chitosan microspheres had a mean diameter of 3.0–4.0 μm. DIC of the suspension, formed after glyoxal crosslinking and before lyophilization, showed that the particles in suspension were spherical. Finally, TEM of the resuspended freeze-dried microspheres showed that the lyophilized chitosan microspheres were spherical.

The average zeta potential values of unloaded and tetracycline-loaded chitosan microspheres are presented in Table 1. The average zeta potential values of unloaded and tetracycline-loaded non-crosslinked chitosan microspheres were +7.45 and +26.68 mV, respectively. For the crosslinked chitosan microspheres, the average zeta potential values of unloaded and tetracycline-loaded microspheres were +5.50 and 21.26 mV, respectively.

Table 1
Average zeta potential values of unloaded and tetracycline-loaded chitosan microspheres

Formulation	Zeta potential (mV)
Unloaded chitosan microspheres	7.45 ± 0.35[a]
Tetracycline-loaded chitosan microspheres	26.68 ± 0.97
Unloaded crosslinked chitosan microspheres	5.50 ± 0.19
Tetracycline-loaded crosslinked chitosan microspheres	21.26 ± 1.07

[a] Mean ± S.E.M. ($n = 3$).

The positive zeta potential values (Table 1) for the unloaded chitosan microspheres (non-crosslinked and crosslinked) could be explained by the fact that most of the amine groups of chitosan having a pK_a of 6.3 (Claesson and Ninham, 1992) are positively charged at pH 3.24 of unloaded chitosan microsphere suspension in deionized distilled water. The zeta potential values for the tetracycline-loaded chitosan microspheres were higher than that of the unloaded chitosan microspheres for both non-crosslinked and crosslinked. This could be explained by the fact that most of the amine groups of chitosan and the tertiary amine group of tetracycline, having a pK_a of 9.69, are positively charged at pH 3.7 of tetracycline-loaded chitosan microsphere suspension in deionized distilled water. Lower zeta potential values for the crosslinked chitosan microspheres either the unloaded or tetracycline-loaded chitosan microspheres, could be explained by the fact that some of the amine groups have been occupied by the chemical crosslinking using glyoxal. The positively charged microspheres are expected to interact with the negatively-charged sialic acid residues of mucin in the stomach by electrostatic interactions and prolong the residence time.

3.2. pH-dependent in vitro drug release studies

For site-specific delivery of tetracycline in the stomach, a pH triggered release system is preferred to allow the drug in solution to diffuse into the mucus layer. The release of the drug from the chitosan microspheres is strongly affected by the pH of medium that can affect the ionization of the glucosamine residues of chitosan. In addition, the pH of the gastric content can vary from a low of 1.2 in the fasted state to a high of 5.0 under fed state or upon concurrent use of antacids. For this reason, tetracycline release kinetics from chitosan microspheres was examined at different pH values. At pH 1.2 and 2.0, the percent tetracycline released was almost the same (53%) and remained at that level after 8 h (Fig. 1). The release profile of the drug at pH 3.5 was higher than that of pH 5.0. Almost 91% of the drug was released after 2 h at pH 3.5 and remained at that level after 8 h, while 79% of the drug

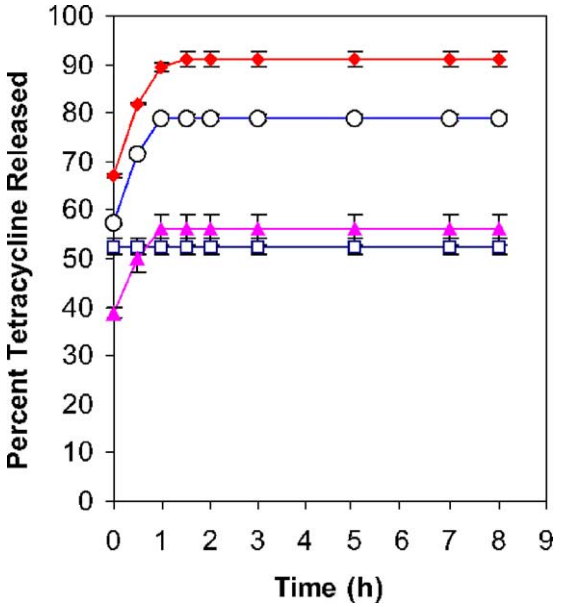

Fig. 1. Percentage of tetracycline released from the microspheres as function of time at 37 °C in different pH medium having ionic strength of 0.16. The symbols represent hydrochloric acid solution: pH 1.2 (□), hydrochloric acid solution: pH 2.0 (▲), acetate buffer: pH 3.5 (◆), and acetate buffer: pH 5.0 (○) (mean±S.E.M., $n = 3$).

was released at pH 5.0 after 2 h and remained at that level after 8 h. Regardless of the pH, the drug was released by burst effect during the first hour and no further release occurred after that. By visual observation of the pellets, the remaining drug indicated by the yellow color was still embedded in the core of the pellets. The microspheres being chemically crosslinked and dense might explain the fact that some of the drug was still entrapped inside the pellets and why not all of the drug was released after 8 h especially at pH 1.2 and 2.0. Visual inspection of the pellets also showed that the pellets formed a gel-like clump at pH 1.2 and 2.0 that might hindered the release of tetracycline and that could explain the fact that the percent released at those pH values was lower than that at pH 3.5 and 5.0. Gel formation al pH 1.2 and 2.0 could be explained by swelling of the chemically crosslinked chitosan because of the ionization of the glucosamine groups at lower pH. Assuming that the drug release profile in vivo does match that seen in vitro, almost 50% of the entrapped tetracycline will be released in the stomach from the microsphere formulation during the first hour after administering orally in fasted conditions. The amount of the drug released will be readily available for absorption and action against *H. pylori*.

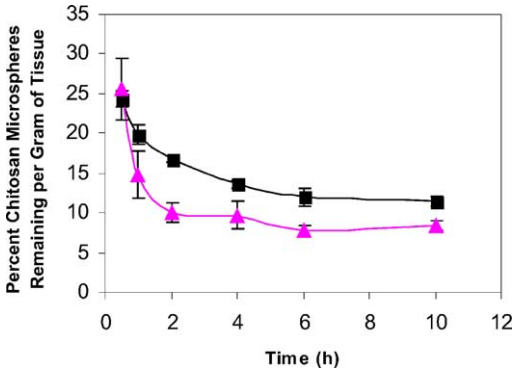

Fig. 2. The gastric residence profile of non-crosslinked (▲) and crosslinked (■) chitosan microspheres after oral administration in gerbils as represented by percent of original dose remaining per gram of tissue as a function of time (mean ± S.E.M., $n = 4$).

3.3. Gastric residence time of chitosan microspheres

3.3.1. Gastric residence of chitosan microspheres

No radioactivity was detected in the phosphate buffer saline rinse. The profile of gastric residence time of chitosan microspheres is shown in Fig. 2. The results are expressed as percent of dose remaining per gram of stomach as function of time. After 2 h in the fasted stomach, approximately 10% of the non-crosslinked chitosan microspheres were present in the stomach and almost remained at that level (8.5%) even after 10 h. However, the percentage remaining of the crosslinked chitosan microspheres in the fasted stomach was 17 and 11.5% after 2 and 10 h, respectively. The mean residence time (MRT), representing the duration for 63.3% of the dose administered to be eliminated (i.e. 36.7% still present in the stomach), was calculated using the ratio of area-under-the-first moment-curve ($AUMC_{0.5\rightarrow 10\,h}$) and the area-under-the-curve ($AUC_{0.5\rightarrow 10\,h}$). As shown in Table 2, the MRT values for the non-crosslinked and crosslinked chitosan microspheres were 4.68 and 4.70 h, respectively. The $AUC_{0.5\rightarrow 10\,h}$ and MRT values of the two groups of animals were compared by two tailed unpaired *t*-test with respect to equality of the variances. The data analysis showed that there was a significant difference between non-crosslinked and crosslinked chitosan

Table 2
Area-under-the-curve ($AUC_{0.5\rightarrow 10\,h}$) and mean residence time (MRT) of chitosan microspheres after oral administration in gerbils[a]

Type of microspheres	$AUC_{0.5\rightarrow 10\,h}$ (percent h/g of tissue)	MRT (h)
Non-crosslinked chitosan microspheres	92.26 ± 11.34[b]	4.68 ± 0.10
Crosslinked chitosan microspheres	132.53 ± 7.19	4.70 ± 0.03

[a] The $AUC_{0.5\rightarrow 10\,h}$ was determined by integrating the percentage remaining as a function of time profile (Fig. 2) after administration of radiolabeled [^{125}I] chitosan microspheres in aqueous suspension to gerbils.
[b] Mean ± S.E.M. ($n = 4$).

microspheres in term of $AUC_{0.5\rightarrow 10\,h}$ (P-value > 0.05). Crosslinked chitosan microspheres profile was higher than that of the non-crosslinked chitosan microspheres for all time points of administration. There was no significant difference between non-crosslinked and crosslinked chitosan microspheres in term of MRT. This could be explained by the fact that the MRT as defined previously represents the last portion of Fig. 2 in which the percent remaining for the non-crosslinked and crosslinked chitosan microspheres was almost the same. For example, After 6 h in the fasted stomach, the percent remaining for the non-crosslinked and crosslinked chitosan microspheres was 8 and 12%, respectively.

3.3.2. Tissue distribution of chitosan microspheres

At 6 h post-administration, we collected the small intestine, colon, kidneys, liver, urine and plasma samples to determine tissue distribution and/or absorption of the microspheres following oral administration. Majority of the microspheres were found in the colon after 6 h as shown in Table 3. There was no detectable

Table 3
Percent of chitosan microsphere dose remaining in the stomach and colon after 6 h in gerbils

Type of microspheres	Percent of dose remaining per gram of tissue	
	Stomach	Colon
Non-crosslinked chitosan microspheres	7.71 ± 0.71[a]	39.4 ± 4.83
Crosslinked chitosan microspheres	12.04 ± 1.13	30.7 ± 1.86

[a] Mean ± S.E.M. ($n = 4$).

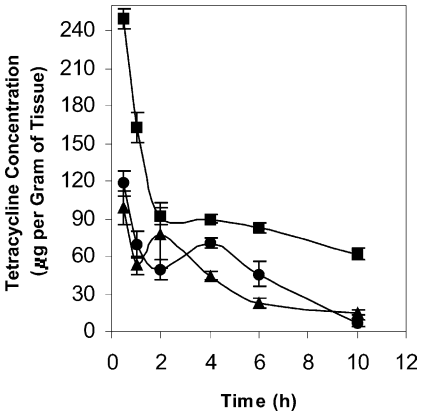

Fig. 3. Tetracycline concentrations in the gerbil stomach represented by microgram of drug per gram of tissue as function of time after administration in solution (●), non-crosslinked (▲), and crosslinked chitosan microsphere (■) formulations (mean ± S.E.M., $n = 4$).

radioactivity in the small intestine, liver, kidney, urine, and plasma samples. The remaining radioactive microspheres were believed to be in the carcass. Based on the absence of detectable radioactivity in the plasma samples, we concluded that these microspheres were not absorbed into the systemic circulation following oral administration.

3.4. Local tetracycline concentrations

In addition to the gastric residence studies of chitosan microspheres, it was important to investigate the correlation between microsphere residence and local drug concentrations. As before, no radioactivity

Table 4
Area-under-the-curve ($AUC_{0.5\rightarrow 10\,h}$) and mean residence time (MRT) of tetracycline in the gerbil stomach after oral administration in Solution and in chitosan microspheres[a]

Formulation	$AUC_{0.5\rightarrow 10\,h}$ (µg h/g of tissue)	MRT (h)
Aqueous solution	447.3 ± 65.0[b]	3.85 ± 0.10
Non-crosslinked chitosan microspheres	358.2 ± 68.7	3.50 ± 0.20
Crosslinked chitosan microspheres	868.9 ± 48.5	4.44 ± 0.01

[a] The $AUC_{0.5\rightarrow 10\,h}$ was determined by integrating the tetracycline concentration as a function of time profile (Fig. 3) after administration of radiolabeled [^{3}H] tetracycline in aqueous solution or in microsphere formulation to gerbils.
[b] Mean ± S.E.M. ($n = 4$).

Table 5
Tetracycline concentrations in the stomach, colon, and urine 6 h after oral administration of solution and microsphere formulation in gerbils

Formulation	Tetracycline concentration (percent of dose per gram of tissue or fluid)		
	Stomach	Colon	Urine
Aqueous solution	3.05 ± 0.68[a]	34.2 ± 1.52	27.2 ± 7.22
Non-crosslinked chitosan microspheres	1.48 ± 0.28	19.8 ± 1.36	18.5 ± 4.50
Crosslinked chitosan microspheres	5.53 ± 0.28	32.8 ± 3.43	22.0 ± 4.89

[a] Mean ± S.E.M. ($n = 4$).

was detected in the phosphate buffer saline wash. Fig. 3 shows the tetracycline concentrations in the stomach, as microgram of drug per gram of tissue, as function of time after administration of an aqueous solution and chitosan microspheres. The bimodal profile of tetracycline concentration in solution is indicative of two different transport mechanisms in the gastric mucosa. When cefuroxime and ciprofloxacin were administered intramuscularly, similar bimodal concentration profile was obtained for each of the drug in the stomach (Westblom and Duriex, 1991). $AUC_{0.5\rightarrow 10\,h}$ and $AUMC_{0.5\rightarrow 10\,h}$ were determined by integrating the tetracycline concentration as a function of time profiles. Table 4 shows the $AUC_{0.5\rightarrow 10\,h}$ and MRT values for the drug solution, non-crosslinked, and the crosslinked chitosan microspheres. The results were compared by two tailed unpaired *t*-test with respect to equality of the variances. The data analysis showed that there were significant differences in the $AUC_{0.5\rightarrow 10\,h}$ and MRT values between the drug solution and the crosslinked chitosan microspheres ($P > 0.05$). Tetracycline concentration profile in the stomach from the crosslinked microspheres was higher than that of the oral solution for all time points of administration. For example, after 0.5 h of administration, the tetracycline concentration for crosslinked chitosan microspheres and drug solution was 249.6 and 118.5 μg/g stomach, respectively. The data analysis also showed that there were significant differences in the $AUC_{0.5\rightarrow 10\,h}$ and MRT values between the crosslinked and non-crosslinked chitosan microspheres ($P > 0.05$). Tetracycline concentration profile in the stomach from the crosslinked microspheres was higher than that of the non-crosslinked chitosan microspheres for all time points of administration. For example, after 4.0 h of administration, the tetracycline concentration for crosslinked and non-crosslinked chitosan microspheres was 89.7 and 44.5 μg/g stomach, respectively.

3.4.1. Tetracycline concentrations in the tissues

At 6 h post-administration, tetracycline was measured in the stomach, colon, and urine samples as shown in Table 5. Interestingly, even though the drug was absorbed into the systemic circulation, we could not detect any of it in the plasma at 6 h. Additionally, there were no detectable tetracycline levels in the small intestine, liver, and kidneys as well.

4. Conclusions

Tetracycline-loaded crosslinked chitosan microsphere formulation was prepared to increase the local concentration of the antibiotic in the stomach and, thus eradicate *H. pylori* infection. The main goal of this study was to examine the gastric residence time of the chitosan microspheres and the local tetracycline concentrations in fasted gerbils. The microspheres remained in the stomach even after 10 h of administration and the $AUC_{0.5\rightarrow 10\,h}$ of the crosslinked microspheres was higher than that of the non-crosslinked microspheres. The chemical crosslinking, using glyoxal, increased the stability of the microspheres under acidic condition and allowed them to settle and adhere to the gastric mucosa by electrostatic interaction. A large fraction of administered chitosan microsphere dose was found in the colon after 6 h. Tetracycline concentration profile in the stomach from the crosslinked microspheres was higher than that of the oral solution or the non-crosslinked chitosan microspheres for all time points of administration. This shows that the crosslinked chitosan microspheres provided significant advantage in increasing the local

concentrations of tetracycline. After 6 h, tetracycline was detected in the stomach, colon, and urine samples. Further studies are planned to develop *H. pylori* infection model in gerbils and to test the efficacy of the tetracycline-loaded crosslinked chitosan microspheres.

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