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Polyelectrolyte Coated Multilayered Liposomes (Nanocapsules) for the Treatment of *Helicobacter pylori* Infection

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Abstract: *Helicobacter pylori* infection is one of the major causes of gastric cancers. A number of systems have already been reported, but 100% eradication has never been achieved. The present invention designs a gastro-retentive drug delivery system incorporated with amoxicillin and metronidazole, specifically suited for the eradication of *Helicobacter pylori* infections due to its mucoadhesiveness in the presence of polyelectrolyte polymers. The system possesses the advantages of both vesicular and particulate carriers, and it was prepared by alternative coating of polyanion (poly(acrylic acid), PAA) and polycation (poly(allylamine hydrochloride), PAH) using liposomes as the core. Compared with the conventional liposomes, the polyelectrolyte based multilayered system (nanocapsules) gave prolonged drug release in simulated gastric fluid, which is well suited for drug delivery against *H. pylori* infection in the stomach. *In vitro* growth inhibition study, agglutination assay, and *in situ* adherence assay in cultured *H. pylori* suggested the successful *in vitro* activity and binding propensity of the system. *In vivo* bacterial clearance study carried out in a *H. pylori* infected mouse model finally confirmed the success of the developed novel nanocapsule system. Thus, the newly developed composite nanocapsules along with the use of combination therapy proved to have commendable potential in *Helicobacter pylori* eradication as compared to already existing conventional and novel drug delivery systems.

Keywords: *Helicobacter pylori*; multilayered liposomes; poly(acrylic acid); poly(allylamine hydrochloride); nanocapsules

1. Introduction

Marshall and Warren in 1979 had discovered the presence of helical shaped bacteria in gastric biopsy samples of a patient's stomach antral regions known as *Helicobacter pylori* which normally resides in the ecological niche of mucus and infects the mucosal lining of the stomach and duodenum and attaches to the gastric epithelial glycolipids. The infection is also associated with gastric and duodenal ulceration.[1] *H. pylori* has been classified as a class I (definite) carcinogen by the International Agency of Research on Cancer (IARC) and World Health Organization (WHO),[2,3] a definite cause of gastric cancers. Development of antibiotic

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resistance due to low concentration and short residence time in the gastric lumen are the major factors for the low eradication rates in conventional single drug therapy.[4] Drug solutions though reach the gastric luminal region, but the mucous layer barrier hinders the absorption of drug in the fundus region of the stomach.

A large number of drug delivery systems are reported such as tetracycline loaded chitosan microspheres,[5] amoxicillin loaded chitosan microspheres,[6,7] sodium alginate microspheres,[8] gelatin microspheres,[9] gliadin nanoparticles,[10] and polyethylcyanoacrylate nanoparticles[11] for the treatment of *H. pylori* infection, but complete eradication could not be achieved. Double and triple therapy based nanoparticles using a combination of drugs to reduce the effective dose required for therapy by achieving synergic and additive effects and to prevent the development of antibiotic resistance have also been reported.[12,13] However, coencapsulation of all the drugs within the same system was not possible; single drug loaded nanoparticles were mixed together to achieve the objectives of multiple therapies, which might have increased the total treatment cost. Liposomes offer the unique advantage over other delivery vehicles in that coencapsulation of more than one drug is readily feasible. However, liposomes are generally unsuitable in practice for oral administration because of their instability during storage and in biological milieu. Here, we propose the development of a stable version of liposomes by coating the conventional liposomes with one or multiple layers of biocompatible polyelectrolytes (alternative coats of polycation and polyanion) in order to stabilize their structure. This new hybrid system combines the advantages of both liposomes as well as nanoparticles.[14,15] This multilayered core−shell structure serves two purposes: the two drugs can be incorporated within the same vesicle, and the multilayered polymer coating helps to provide a robust physical structure to protect the vesicle core.

Further, the combination of phospholipids and mucoadhesive polymers with a *H. pylori*-eradicating antibiotic also created a surprisingly synergistic effect. The phospholipids−antibiotic composite is specifically carried to the desired site of action, where it decomposes and releases the drug on the surface of the mucosa and forms a protective layer. The protection of entrapped drugs from luminal degradation depends mainly on the physical integrity of the liposomes and then on the permeability of the bilayer to the gastric environment. Though the mechanism of gastric mucosal damage due to *Helicobacter pylori* is not very clear, the phospholipids provide fatty acids for repair of the damaged mucosa.[16] Compared with other delivery systems, the polyelectrolyte based multilayered system is expected to give a sustained drug release in the stomach, which is well suited for treatment of *H. pylori* infection. In this study, we have tried to use layer-by-layer assembly to allow polyelectrolyte adsorbed onto an oppositely charged surface of lipid vesicles to stabilize the liposomes.[15,17,18] The positively charged outer layer of liposomes is believed to help in the attachment of the hybrid system to the negatively charged mucosal surface.

In the present study, a combination of amoxicillin and metronidazole was taken to overcome the resistance development problem associated with the monotherapies. The presence of two drugs in the same system would lead to more improved efficacy and increased patient compliance.

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2. Materials and Methods

2.1. Materials. Egg phosphatidylcholine (PC), cholesterol (CH), stearylamine (SA), Sephadex G-50, poly(allylamine hydrochloride) (PAH, MW ∼ 56 kD), and fluorescein-5-isothiocyanate (FITC) were purchased from Sigma Aldrich, St. Louis, MO. The drugs amoxicillin trihydrate and metronidazole were obtained as gift samples from Promise Pharmaceuticals, Sagar (MP) India. Poly(acrylic acid) (PAA, MW ∼ 50 kD) was procured from Himedia Laboratories, Mumbai, India. Dehydrated Brucella broth and Brucella chocolate agar were obtained from Difco Laboratories, Detroit, MI, and fetal calf serum (FCS) used was obtained from Gibco BRL, Uxbridge, U.K. The E-test strips were purchased from AB Biodisk, Solna, Sweden.

2.2. Preparation of Positively Charged Liposomes. Positively charged liposomes consisting of PC/CH/SA in a molar ratio of 7:3:1 were prepared by lipid film hydration technique.[19] The lipid components (72 mg) were dissolved in chloroform/methanol mixture (2:1 v/v) in a round-bottom flask. The amphiphilic drug metronidazole (5 mg) was dissolved in methanol and added to the lipid phase. The organic solvent was evaporated under reduced pressure using a Buchi rotary flash evaporator to cast the thin lipid film on the walls of the round-bottom flask. Lipid film hydration was done using amoxicillin trihydrate (10 mg) in phosphate buffered saline (5.0 mL, 0.01 M, pH 7.4). The prepared liposomes were sonicated with the help of a probe sonicator (Soniweld, India) for 5 min at 40 Kc/s to reduce the vesicle size and finally passed through a 0.20 μm membrane filter to obtain small sized vesicles. The prepared liposomal dispersion was passed through a Sephadex G-50 column to remove the unentrapped drug. The percent entrapment was determined and expressed as the percentage fraction of the drug incorporated in the vesicles with respect to the overall drug used in vesicle preparation. Vesicles were lysed with a minimum amount of Triton X-100 (0.5%, v/v) and centrifuged, and the liberated drugs were analyzed using a Shimadzu 1601 UV/visible spectrophotometer against a reagent blank at λ_{max} 272 nm and 320 nm for amoxicillin trihydrate and metronidazole, respectively. The absorbance data for both wavelengths were treated using simultaneous equations generated (equations not shown) to calculate the amount of drug in each sample. All of the above-mentioned operations were carried out under aseptic conditions to maintain the sterility.

2.3. Coating of Liposomes (Preparation of Multilayered Liposomes/Nanocapsules). The layer-by-layer process described previously for multilayer polyelectrolyte films[20] was applied to liposomes to produce the double layer.[14,18] The polyelectrolyte solutions were prepared at 0.25% w/v poly(acrylic acid) (PAA) and 0.5% w/v poly(allylamine hydrochloride) (PAH). The first layer of the negatively charged polymer, that is, PAA (100 μL), was adsorbed over the surface of positively charged liposomes (10 mL, 5 mM lipids). Excess polymers were removed using high-speed centrifugation (15 000 rpm for 20 min) and a dialysis bag (70 kD, Sigma Aldrich, St. Louis, MO). Subsequently, a layer of PAH was coated in a similar manner as described above. The particle size and zeta potential were checked using a Malvern zetasizer, and the zeta potential was found to reverse after each coat.[18,21] The surface morphology of the nanocapsules formed was ascertained using transmission electron microscopy (TEM, Philips, Japan).

2.4. Drug Release Study. The *in vitro* drug release profile of prepared multilayered liposomes (nanocapsules) was performed in simulated gastric fluid (SGF, pH 1.2), which was prepared by dissolving 2 g of sodium chloride (NaCl) in about 250 mL of distilled water followed by addition of 3.2 g of pepsin dissolved in 7 mL of concentrated HCl. Distilled water was then added to make up the volume up to 1 L, and the pH of the resultant solution was adjusted to 1.2. Formulations (1 mL) were filled in already treated dialysis bags. Assembly was arranged in a beaker containing 500 mL of release media. The beaker was placed over a magnetic stirrer to maintain sink conditions, and the temperature was maintained at 37 ± 1 °C. Samples were pipetted out after regular time intervals for up to 24 h. An equal volume of the fresh media was replaced to maintain the volume. The drug content in each sample was determined spectrophotometrically using a Shimadzu 1601 UV/visible spectrophotometer as described above.

2.5. Culture of *Helicobacter pylori* Isolates. Brucella chocolate agar media was prepared by sterilizing ready made media after dissolving in distilled water followed by addition of 10−20% freshly collected sheep blood. To this media, vancomycin 6 mg/mL, amphoterecin B 2 mg/mL, and polymyxin B sulfate 2500 IU/mL were added. Human biopsy samples, which were collected from Department of Gastroenterology, S.G.P.G.I.M.S., Lucknow (U.P.) India, were swabbed on Brucella chocolate agar media. Plates were incubated in a candle jar at 37 °C under microaeropfhilic environment (80% N_2, 15 CO_2, 5%O_2) with a pad of cotton soaked with water to maintain 90−100% humidity. The plates were examined after 72 h to see the colony growth. The organism was identified on the basis of modified Gram staining, oxidase, catalase, and urease tests.[22] The colonies were found to be translucent and gray with size range of 0.5−1 μm. The bacteria were then subcultured in Brucella broth medium. The cultured *H. pylori* strains could be stored either at −70 °C in normal saline or in liquid nitrogen or in Brucella broth in 20% glycerine.

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2.6. Antibiotic Susceptibility Assay by E-Test. The method reported in the literature was used for the E-test.[23,24] The E-test (epsilometer test) strips (AB Biodisk, Solna, Sweden) were used for determination of minimum inhibitory concentration (MIC) of antibiotics used in the study against *H. pylori*. Agar plates of 140 mm diameter were inoculated with *H. pylori* suspension (McFarland tube no. 3) by swabbing of the surface. E-test strips were aseptically placed onto the dried surfaces of each inoculated agar plate. The plates were incubated at 37 °C under microaerophilic conditions and 100% relative humidity. The MIC was determined after the intersection of the elliptical zone of inhibition with the MIC scale on the E-test strip (as per the manufacturer's instructions) after 72 h of incubation. The isolates were considered resistant when the MIC value was greater than the susceptible break point of greater than 8 μg/mL for metronidazole[25] and greater than 2 μg/mL for amoxicillin.[26]

2.7. *In Vitro* Growth Inhibition Studies. Equal volumes of suspension of *H. pylori* (20 mL each) in Brucella broth were prepared (with 10% FCS) and filled in different culture flasks. The formulations of amoxicillin and metronidazole were also added to them in calculated amounts to get the dose equivalent to MIC, and the flasks were incubated in microaerophilic environment at 37 °C. The bacterial growth was monitored by optical density (OD) measurement[10] using a Shimadzu 1601 UV/visible spectrophotometer at 660 nm against blank, that is, uninoculated, broth daily for up to 4 days. Culture flasks containing placebo systems were also included in the study so as to detect any antimicrobial activity due to the lipids and the polymers used in preparation of the system.

The percent growth inhibition was determined using the formula:

$$\%\text{ growth inhibition} = \frac{\text{OD of control} - \text{OD of sample} \times 100}{\text{OD of control}}$$

where control refers to *H. pylori* broth and sample refers to *H. pylori* broth incubated with formulations.

2.8. *In Vitro* Agglutination Assay. *Helicobacter pylori* (strains HP 1753/PGI and HP 1756/PGI) were grown on Brucella chocolate agar media and then subcultured in Brucella broth liquid medium at 37 °C in microaerophilic environment for 4−5 days. A pure culture was obtained after two to three passages. A suspension of the cultured bacteria (having known turbidity) was mixed with drug free formulations (1/10 volume) in a sterile Eppendorf tube and kept at 37 °C for 1 h in a candle jar. Pure *H. pylori* suspension was taken as the control. A drop of suspension from each of the tubes was added to a glass slide, and a smear was prepared. The smear was then stained using a modified Gram-negative stain and visualized under a microscope (LEICA, Germany) to observe any agglutination.

2.9. *In Situ* Adherence Assay. The *in situ* adherence assay is useful in characterization of *H. pylori* adhesin and receptors as well as for identifying useful drugs that inhibit strain specific binding of this human pathogen. This assay also helps in determining the targeting propensity of different drug delivery systems to *Helicobacter pylori*. The protocol followed was the same as that reported and validated earlier[27] for determining the *in situ* adherence of the *H. pylori* with the liposomes and nanoparticles.[28]

Healthy, disease free male Balb/c mice (weight = 25−35 g and age = 6−8 weeks) were procured from the animal house of I.T.R.C., Lucknow (U.P.), India. All the animal study protocols were duly approved by the Institutional Animal Ethics Committee, and the studies were carried out in accordance with the Council for Purpose & Supervision of Experiment on Animals (CPCSEA), Ministry of Social Justice & Empowerment, Government of India. The mice were kept with proper maintenance of temperature (25 °C), relative humidity (55%), and light/dark cycle (12 h/12 h). They were provided appropriate feed and continuous water supply of fresh water. Gastric tissues of the mice were collected after sacrifice by cervical dislocation and regional dissection. All tissues were fixed in 10% formalin and subsequently embedded in paraffin. Microtomy was performed, and thin sections of tissue (~5 μm) were mounted on glass slides.

Helicobacter pylori collected from human stomach biopsy samples were grown on Brucella chocolate agar media as described previously, and bacteria from pure culture were suspended in 0.1 M NaCl (sterile) by gentle pipeting. A 10 μL aliquot of a 10 mg/mL solution of FITC in dimethyl sulfoxide was added to this suspension and allowed to incubate at 37 °C for 1 h in dark. The bacterial suspension was centrifuged at 8000 rpm for 10 min to get the pellet. The pellets were washed thrice, resuspended in PBS (pH 7.4, 0.1 M), and then stored at −70 °C for further use. The tissue sections were deparaffinized for 15 min in xylene and then for 30 min in isopropyl alcohol. Tissues were then rinsed in water and finally incubated in blocking buffer (0.2% bovine serum albumin/0.05% Tween 20/PBS, pH 7.4). The FITC labeled bacterial suspension was then diluted with blocking buffer, and 200 μL of this suspension was placed

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over the tissue sections and kept at room temperature for 2 h in a humidified chamber.

For determining *in situ* binding in the presence of liposomes and nanocapsules, the FITC labeled bacteria were preincubated with an equal amount of different drug free formulations for 2 h prior to their incubation over the tissue sections. The slides were washed six times with PBS, visualized under a fluorescent microscope (OLYMPUS BX60, Germany), and photomicrographs were taken.

2.10. *In Vivo* Clearance Study. The method followed for *in vivo* study was Qian's method (China patent, CN 1304729A). Healthy, disease free male Balb/c mice (weight = 25−35 g and age - 6−8 weeks) were used for the study. After an overnight fasting, mice were inoculated with an equal amount of bacterial suspension (1 mL) by intragastric gavage using a sterile gastric canula such that each dose contained approximately log 7.7 ± 0.47 colony forming units (CFU) of *H. pylori*. The dosing was done once daily for 3 consecutive days to make them properly infected. After the development of infection after 2 weeks, the mice were randomly divided in different groups with each containing five mice and different formulations were administered once daily for three consecutive days as per the following dosing schedule:

1. Sterile water (control group)
2. Solution containing both drugs, dose equivalent to 10 mg/kg amoxicillin and 5.50 ± 0.05 mg/kg metronidazole
3. Solution containing both drugs, dose equivalent to 20 mg/kg amoxicillin and 11.0 ± 0.05 mg/kg metronidazole
4. Solution containing both drugs, dose equivalent to 30 mg/kg amoxicillin and 16.5 ± 0.12 mg/kg metronidazole
5. Nanocapsules containing both drugs, dose equivalent to 10 mg/kg amoxicillin and 5.50 ± 0.05 mg/kg metronidazole
6. Nanocapsules containing both drugs, dose equivalent to 20 mg/kg amoxicillin and 11.0 ± 0.05 mg/kg metronidazole
7. Nanocapsules containing both drugs, dose equivalent to 30 mg/kg amoxicillin and 16.5 ± 0.12 mg/kg metronidazole
8. Plain liposomes containing both drugs, dose equivalent to 10 mg/kg amoxicillin and 5.50 ± 0.05 mg/kg metronidazole
9. Plain liposomes containing both drugs, dose equivalent to 20 mg/kg amoxicillin and 11.0 ± 0.05 mg/kg metronidazole
10. Plain liposomes containing both drugs, dose equivalent to 30 mg/kg amoxicillin and 16.5 ± 0.12 mg/kg metronidazole
11. Solution containing both drugs, dose equivalent to 30 mg/kg amoxicillin and 16.5 ± 0.12 mg/kg metronidazole, plus blank nanocapsules

One day after the administration of the final dose, the mice were killed and stomachs were excised. A part of the stomach was used for histopathological study using Hematoxylin-Eosin staining. The remaining stomach was homogenized in homogenizer with 3 mL of Brucella broth. Brucella broth was then tested to confirm the presence of urease using a rapid urease test, and *Helicobacter pylori* were observed in a 1000× microscope after modified Gram staining. Serial dilutions were made, and the bacteria were finally grown on Brucella chocolate agar media at 37 °C in microaerophilic conditions for 4−5 days. A viable bacterial colony count per plate was done and reported in log CFU per gram of gastric tissue. The effect of multilayered core−shell structures with the combination of amoxicillin and metronidazole was then reported on the basis of log CFU and also clearance rate.

2.11. Statistical Analysis. The results were expressed as mean ± standard deviation (SD). Differences between the control and drug loaded formulation treated groups in bacterial colony count in the gastric wall were statistically analyzed by ANOVA, followed by Dunnet's multiple comparison test, as post test ($n = 5$). Statistical significant differences between groups were defined as $P < 0.05$. Calculations were performed with the Graph Pad Instat software program (Graph Pad Software Inc., San Diego, CA).

3. Results

3.1. Preparation and *In Vitro* Characterization. Positively charged liposomes containing amoxicillin and metronidazole were successfully prepared by cast film technique. Initial experiments were conducted to optimize the coating procedure. The first coat of anionic polymer PAA was applied onto the liposomes. Figure 1 shows the effect of polymer concentration on zeta potential and vesicle size. On increasing the polymer concentration for coating, the particle size was found to increase and at the same time the positive value of the zeta potential fell down steeply and then started increasing in the negative direction and became constant (−18.58 ± 0.08 mV) at PAA concentration 0.25% (Figure 1), which was considered as the optimum concentration for complete coating. In the case of the second coating with cationic PAH, the zeta potential value increased up to 0.5% polymer concentration and then became constant (Figure 1). The particle size, zeta potential, polydispersity index, and drug encapsulation efficiency of various formulations are given in Table 1.

The coating of polyelectrolytes over liposomes can be clearly seen in TEM photomicrographs, which revealed that the transparent liposomes (photograph not shown) became opaque due to polymer coating (Figure 2). The surface morphology of the coated system was similar to that of the particulate system, showing that nanocapsules were formed after coating of the liposomes. Without coating, the liposomes appeared as white spots. After coating, a dark rim corresponding to the polyelectrolyte capsule stained by phosphotungstic acid can be seen.[12] The coating thickness was also determined by TEM and was found to be ∼24−30 nm.

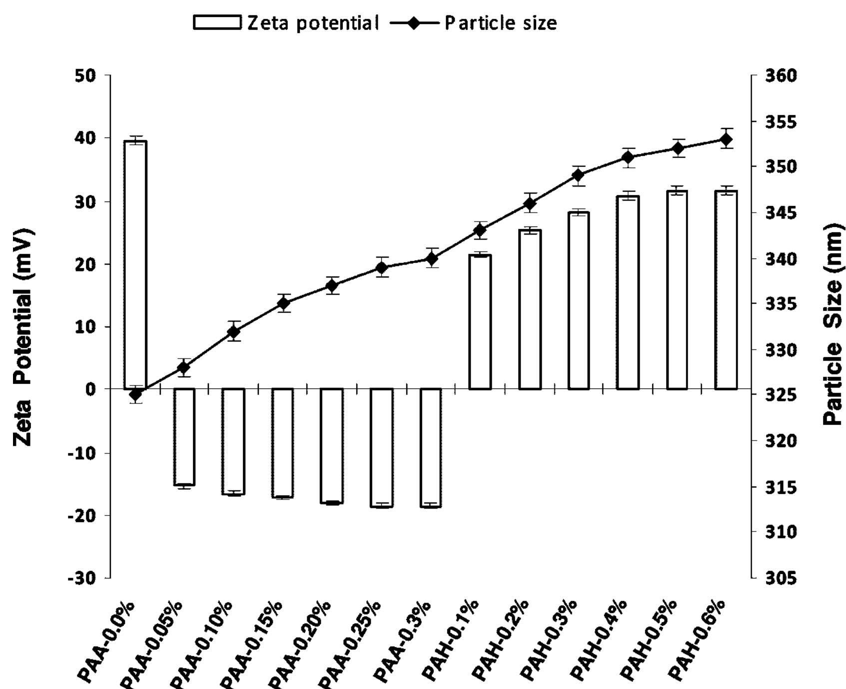

Figure 1. Effect of polymer concentration on zeta potential and particle size.

Table 1. *In Vitro* Characterization of the Developed Formulation[a]

serial no.	formulation	zeta potential (mV)	mean particle size (nm)	polydispersity index (PDI)	% entrapment efficiency (amoxicillin)	% entrapment efficiency (metronidazole)
1	plain cationic liposomes	+39.60 ± 0.50	325 ± 12	0.113 ± 0.031	48.38 ± 3.42	53.75 ± 3.53
2	PAA coated liposomes	−18.58 ± 0.08	339 ± 17	0.123 ± 0.024	48.17 ± 3.56	53.34 ± 3.64
3	PAH coated liposomes (nanocapsules)	+31.64 ± 0.37	352 ± 21	0.145 ± 0.018	47.71 ± 3.65	52.14 ± 3.58

[a] Values are shown as mean ± SD ($n = 6$).

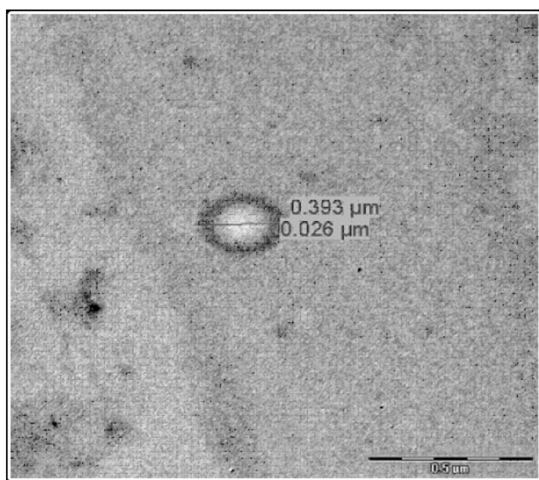

Figure 2. TEM photomicrograph of polyelectrolyte coated multilayered liposome (nanocapsule).

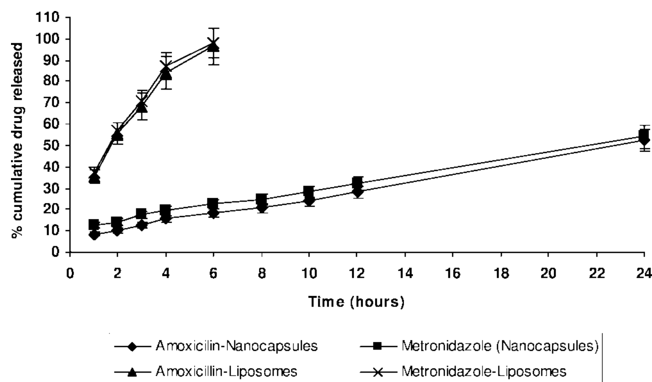

Figure 3. Cumulative percent of drug release from plain and polyelectrolyte coated multilayered liposomes (nanocapsules).

3.2. *In Vitro* Drug Release. Figure 3 shows the cumulative percent drug release of amoxicillin trihydrate and metronidazole from drug loaded nanocapsules in SGF (pH 1.2). Multilayered liposomes (nanocapsules) showed a significantly slow release profile as compared to plain liposomes. The cumulative percent release of 52.3 ± 4.6% amoxicillin and 54.1 ± 5.1% metronidazole was observed in SGF after 24 h from nanocapsule formulations, while plain liposomes released >95% drug within 6 h only.

3.3. Culture of *Helicobacter pylori* Strain. It was necessary to culture the *Helicobacter pylori* strains in a microaerophilic environment, as in the presence of excess oxygen coccoidal forms may develop which are resistant to growth. For the present study, the strain of *H. pylori* was isolated from a patient with a duodenal ulcer in the Department of Gastroenterology, S.G.P.G.I.M.S, Lucknow, India. The isolated strain was grown on Brucella chocolate agar media

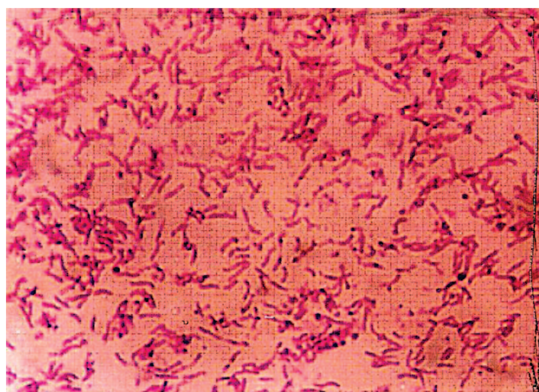

Figure 4. Gram stained *Helicobacter pylori* (40×).

Table 2. Determination of MIC of Drugs for *Helicobacter pylori* by E-Test

serial no.	drug	sensitive/resistant	MIC
1	amoxicillin trihydrate	sensitive	0.015 μg/mL
2	metronidazole	sensitive	256 μg/mL
3	amoxicillin trihydrate + metronidazole	sensitive	<0.015 μg/mL

(DIFCO, Detroit, Mich.) having 20% sheep blood and also in Brucella broth liquid media in the Department of Microbiology, S.G.P.G.I.M.S., Lucknow. Sheep blood was used in the present study, as it is believed to support excellent organism growth.

The *Helicobacter pylori* colonies were found to be translucent, gray, and shiny in morphology, with an average cell size of 2−4 μm. The colony appeared as tiny water droplets on a solid agar plate. On repeated subculturing and in the presence of aerobic conditions, the coccoidal form appeared in the culture.

Modified Gram staining of the biopsy samples showed the color change of the media from yellow to pink. Gram staining also helped in observing the shape of the bacteria, which was rodlike or spiral in shape (Figure 4). Cultured *H. pylori* gave positive urease, oxidase, and catalase tests.

3.4. Antibiotic Susceptibility Assay. It was important to determine the susceptibility of antibiotics to a particular strain for the selection of antibiotics and also for the optimization of dose. By disk diffusion assay, it was found that the *H. pylori* strain was sensitive to both amoxicillin and metronidazole. By the E-test method, the MIC values were found to be 0.015 μg/mL for amoxicillin and 256 μg/mL for metronidazole, and when both antibiotics were combined in a formulation, the MIC was found to be less than 0.015 μg/mL (Table 2). This method of MIC determination was found to be more accurate in the case of fastidious organisms such as *H. pylori*, as compared to disk diffusion assay, but it is more expensive.[29]

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3.5. *In Vitro* Bacterial Growth Inhibition Study. The maximum growth inhibition observed after 4 days with plain drugs amoxicillin and metronidazole was found to be 99.04 ± 1.16% and 98.74 ± 1.23%, respectively (Table 3). It was observed that amoxicillin gave better antimicrobial activity as compared to metronidazole, but a synergistic effect was observed when both drugs were mixed together as the mixture was able to inhibit 99.64 ± 0.56% growth of the bacteria after 4 days.

The growth inhibition was found to be 94.97 ± 2.37% and 94.61 ± 2.40% for the placebo liposomes and nanocapsules (without any drug), respectively, while the same formulations containing drugs exhibited growth inhibition of 98.38 ± 1.15% and 99.04 ± 1.12%, respectively. These results suggested that the plain drugs inhibited the bacterial growth *in vitro* at a relatively faster rate as compared to drug containing formulations as the systems released the drugs slowly and steadily, so lesser amounts of drugs were exposed to the bacteria, while free drugs had direct exposure to the bacteria.

3.6. *In Vitro* Agglutination Assay. Table 4 presents the agglutination reaction which occurred on incubating the bacteria with drug free formulations. Among the various formulations, for example, liposomes, nanocapsules (single, PAA coated), nanocapsules (double, PAA and PAH coated), and plain phosphatidylcholine, highest agglutination was found when nanocapsules (coated with both PAA and PAH) were incubated with *H. pylori*. Agglutination was also seen when *Helicobacter pylori* was incubated with PAA coated liposomes (single nanocapsules) and plain liposomes. Agglutination reaction shows the ability of bacteria to agglomerate together due to adhesin receptors present on their cell surface.

3.7. *In Situ* Adherence Assay. *In situ* adherence assay was done by using fluorescent labeled bacteria (FITC used) that were incubated with various drug free formulations. Fluorescent photomicrographs were taken, and the number of bacteria showing fluorescence was compared with the control group (Figure 5a). The pure *H. pylori* adhered well to the gastric mucosal surface as observed in Figure 5a. The *Helicobacter pylori* strains, which were treated with liposomes, could not bind efficiently to the gastric epithelial cells (Figure 5b), which might be due to the effect of phosphatidylcholine on the binding propensity of *Helicobacter pylori*. The phosphatidylethanolamine receptors present on the gastric cells that were somewhat bound to the liposomes, which contained phosphatidylcholine as a major constituent, did not allow binding of *H. pylori* to gastric cells.

A plug and seal effect was observed in the case of treatment with nanocapsules, which further inhibited the mucosal binding of bacteria (Figure 5c). The reason behind this might be the relatively more dense layer formed by the polymers and binding of positively charged polymers on the negatively charged mucosal surface as compared to FITC labeled bacterium. It has been also found that similar to

Table 3. *In Vitro* Bacterial Growth Inhibition Offered by Various Formulations[a]

serial no.	formulation	percent growth inhibition			
		day 1	day 2	day 3	day 4
1	BB + HP + Amox	60.00 ± 2.34	98.24 ± 2.12	97.45 ± 2.45	99.04 ± 1.16
2	BB + HP + Metro	73.33 ± 2.54	97.36 ± 2.22	97.77 ± 2.34	98.74 ± 1.23
3	BB + HP + Amox + Metro	73.33 ± 3.14	97.36 ± 2.62	98.08 ± 2.82	99.64 ± 0.56
4	BB + HP + plain Lipo	60.0 ± 2.78	96.49 ± 2.55	94.26 ± 2.28	94.97 ± 2.37
5	BB + HP + plain Nano	46.66 ± 2.32	86.84 ± 2.46	92.99 ± 2.65	94.61 ± 2.40
6	BB + HP + Lipo + Amox	53.33 ± 2.56	88.59 ± 2.60	94.58 ± 2.74	98.02 ± 1.91
7	BB + HP + Lipo + Metro	53.33 ± 1.23	87.71 ± 2.63	94.90 ± 2.90	96.05 ± 2.23
8	BB + HP + Nano + Amox	46.66 ± 1.90	85.96 ± 2.57	93.94 ± 2.61	97.84 ± 1.90
9	BB + HP + Nano + Metro	60.0 ± 2.45	87.71 ± 2.34	94.58 ± 2.40	97.85 ± 1.88
10	BB + HP + Lipo + Amox + Metro	60.0 ± 2.18	90.35 ± 2.72	96.49 ± 2.44	98.38 ± 1.15
11	BB + HP + Nano + Amox + Metro	46.66 ± 2.24	89.47 ± 2.48	93.63 ± 2.56	99.04 ± 1.12

[a] BB = Brucella broth; HP = *Helicobacter pylori*; Nano = nanocapsules; Lipo = liposomes, Amox = amoxicillin trihydrate; Metro = Metronidazole. Values are shown as mean ± SD ($n = 5$).

Table 4. Agglutination Pattern of Various Formulations for the *H. pylori* Strain[a]

serial no.	formulation	agglutination[b]
1	liposomes	++
2	nanocapsules (PAH coated)	++++
3	phosphatidylcholine	+

[a] *H. pylori* culture taken as control. [b] The results of agglutination assay are given as follows: ++++ = strong positive reaction with large clumps; ++ = strong positive reaction with moderate size clumps; + = positive reaction with fine clumps.

polysaccharides[30] mucoadhesive polymers such as PAA and PAH also act as an antiadhesive agent against *H. pylori* colonization of gastric mucin.

3.8. *In Vivo* Clearance Study. The histopathology of the gastric mucosa of mice infected with *H. pylori* is shown in Figure 6. *H. pylori* infection is clearly visible in the photomicrograph (Figure 6). The *in vivo* clearance data of *H. pylori* infections after multiple administrations of various formulations bearing amoxicillin and metronidazole under fed conditions are presented in Table 5. The mean bacterial count (log colony forming units; log CFU) of the control group of mice, which were given only sterile water, was found to be log 6.82 ± 0.13 CFU. The mean bacterial count of the bacteria after oral administration of plain drugs decreased upon increasing the drug dose, but complete eradication was not achieved even with the highest dose (30 mg/kg amoxicillin and 16.5 ± 0.12 mg/kg metronidazole). Treatment with plain drug solution in a dose of 10 mg/kg amoxicillin and 5.50 ± 0.05 mg/kg metronidazole gave a mean bacterial count of log 5.49 ± 0.87 CFU. A higher drug dose (30 mg/kg amoxicillin and 16.5 ± 0.12 mg/kg metronidazole) resulted in a colony count of log 1.84 ± 0.24 CFU with only 60% clearance rate, which might be due to the unavailability of 100% drug and short residence time of drugs in the stomach and the low concentration of the drugs reaching the bacteria under the gastric mucus layer.

The mean bacterial colony count after oral administration of liposomes containing drug in a dose equivalent to 20 mg/kg amoxicillin and 11.0 ± 0.05 mg/kg metronidazole was 1.48 ± 0.47. The formulation gave only 80% clearance rate, while the colony count in the case of nanocapsules with the same dose was below the detection limit (≤log 1.0 CFU) in all the animals; no sign of *H. pylori* infection was detected in the animals. It means treatment with drug loaded nanocapsules in a drug dose of 20 mg/kg amoxicillin and 11.0 ± 0.05 mg/kg metronidazole or higher resulted in complete eradication of *H. pylori* infection and thus the mucoadhesive nanocapsules were found to be effective in the treatment of *Helicobacter pylori* infections.

In the *in vitro* studies, blank or empty nanocapsules have also demonstrated significant antibacterial activity. So, to test whether the 100% clearance rate observed with drug loaded nanocapsules was due to encapsulation of drugs into nanocapsules or due to combined the antibacterial effect of drug and nanocapsules, we tested the mixture of plain drugs in the highest doses along with blank empty nanocapsules. In this case, only 60% clearance as achieved with the same dose of plain drug mixture given alone was obtained. The reduction in mean colony count was although lower as compared to plain drugs (1.65 ± 0.21), but it was statistically insignificant ($P < 0.05$).

4. Discussion

The rationale for developing this drug delivery system was the need of a carrier system which could deliver the drug to the desired site of action in therapeutic concentrations over a prolonged period of time. Polyelectrolyte based multilayered liposomes (composite nanocapsules) combine the advantages of vesicles by incorporation of two drugs in the same system and also have a rigid and robust outer polymeric layer (such as particulates) to protect the vesicles in the harsh gastric environment. Yokel and co-workers[31] had proposed that on increasing the contact time of the antibiotic with the bacteria the eradication rate could be improved. Mucoadhesive drug delivery systems thus provide sustained drug release by localizing the drugs in the mucosal region where

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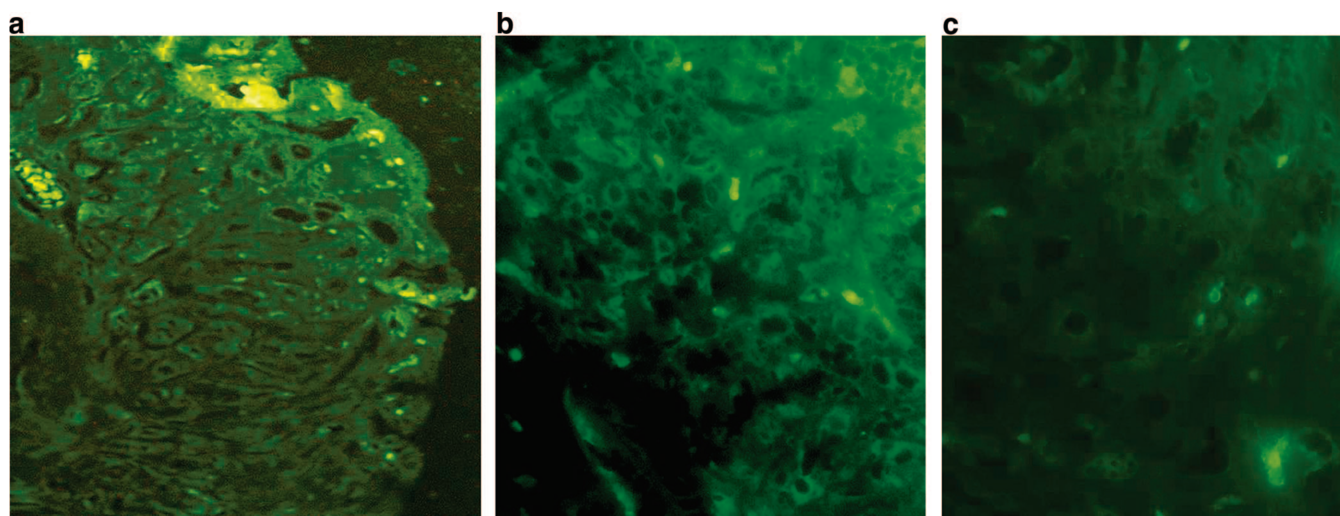

Figure 5. Fluorescence image of mice gastric mucosa showing *in situ* adherence of FITC labeled *H. pylori* (40×): (a) pure *H. pylori* (control), (b) treated with liposomes, and (c) treated with nanocapsules.

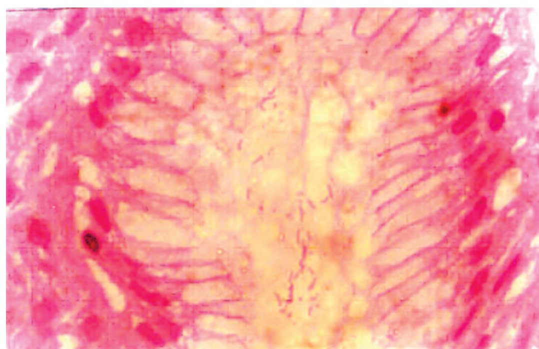

Figure 6. Histopathology of mice gastric mucosa after *H. pylori* infection (40×) (using Hematoxylin-Eosin stain).

the bacteria reside.[32,33] This would also increase the antibiotic concentration in the luminal region of the stomach by promoting the diffusion of the antibiotics in the epithelial cell layer and thus would be effective in inhibiting the growth of bacteria in that region. Similar to positively charged chitosan, poly(allylamine hydrochloride) (PAH) is also expected to neutralize the hydronium ions present in gastric acid by producing NH_3^+ ions.[34] The template core used in the preparation of nanocapsules can be DNA, enzymes, proteins, biologicals, and vesicular and particulate carrier systems. We used liposomes as the core in our study because liposomes have been found to be one of the most versatile drug delivery systems in novel drug delivery, and both amoxicillin, which is a hydrophilic drug, and metronidazole, which is an amphiphilic drug, can be incorporated in the same system. Moreover, liposomes form a protective hydrophobic coat around the antibiotics which prevents them from degradation in the gastric milieu.[35] The lipids of the liposomes not only allow the drugs to be carried to the desired site of action but also help in reducing the side effects of the antimicrobials released. Liposomes also provide fatty acids for the repair of the damaged epithelial membrane due to *Helicobacter pylori* infection.[36]

The presence of surfactants causes degradation of liposomes which cannot be avoided especially in gastric conditions. Thus, the polymeric coating seems to be very useful in keeping the vesicles intact to release the drugs at a controlled rate. This was very well reflected in an *in vitro* drug release study in SGF (pH 1.2) where the nanocapsules exhibited 52−55% cumulative drug release after 24 h in comparison to liposomes which released >95% of encapsulated drug within 6 h only. Also, the polymers used were mucoadhesive in nature, making the system mucoadhesive. PAA is negatively charged, but it is reported to have high interaction with the gastric mucosa because of the physical entanglement with the cell surfaces. The polymeric coating over liposomes prevented back-diffusion of the acid in the stomach. PAH is a biomimicking polymer with an NH_2 functional group and has been vigorously studied for PAA/PAH multilayer formation due to the strong effect of salt concentration and pH on the conformational and mechanical properties. However, the dynamic behavior of polyelectrolytes is much more complicated than that of the uncharged

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Table 5. *In Vivo* Bacterial Clearance in *H. pylori* Infected Mice[a]

serial no.	treatment given	dose (mg/kg)	clearance rate (no. of mice cleared with infection/total no. of mice), %	mean bacterial colony count (log CFU ± SD)
1	sterile water (control)		0/5 (0)	6.82 ± 0.13
2	Plain drug solution (Amox + Metro)	10 + 5.5	2/5 (40)	5.49 ± 0.87
3		20 + 11	3/5 (60)	4.26 ± 0.66
4		30 + 16.5	3/5 (60)	1.84 ± 0.24
5	liposomes (Amox + Metro)	10 + 5.5	3/5 (60)	2.51 ± 1.12
6		20 + 11	4/5 (80)	1.48 ± 0.47
7		30 + 16.5	4/5 (80)	ND
8	nanocapsules (Amox + Metro)	10 + 5.5	4/5 (80)	1.38 ± 0.39
9		20 + 11	5/5 (100)	ND
10		30 + 16.5	5/5 (100)	ND
11	plain drug solution (Amox + Metro) + blank nanocapsules	30 + 16.5	3/5 (60)	1.65 ± 0.21

[a] CFU = colony forming unit; Amox = amoxicillin trihydrate; Metro = metronidazole, ND = not detected (CFU ≤ 1.00). Values are shown as mean ± SD ($n = 5$).

polymers due to the multiplicity of interactions present in the polyelectrolyte solution, the nature of which is not fully understood.

During the coating process, oppositely charged polymer gets adsorbed and coated onto the surface of vesicles due to electrostatic interaction and resulted in reversion of the zeta potential after each coat (Table 1). In this study, preformed drug loaded liposomal vesicles were coated with negatively and positively charged polymers. This is presumably the reason for the insignificant changes recorded in the entrapment efficiency values (Table 1) after coating.[21]

H. pylori obtained from the human biopsy sample was cultured in a microaerophilic environment to prevent development of growth resistant coccoidal forms, which may like to develop in the excess of oxygen. The bacteria were identified using modified Gram staining and also gave positive urease, oxidase, and catalase tests. The minimum inhibitory concentration of the bacteria was determined by E-test. This method of MIC determination was found to be more accurate in the case of fastidious organisms such as *H. pylori*, as compared to the disk diffusion assay,[29] but it is more expensive.

The turbidometric method was used for performing the growth inhibition studies. In this study, the percentage growth inhibition of the test mixture was compared to that of pure *Helicobacter pylori* culture on the basis of the optical density measurement method.[33] The growth of the bacteria caused an increase in turbidity of the culture media. However, when the formulations were added to the culture flasks during incubation under microaerophilic conditions, the increase in turbidity was checked by antibiotics. Both amoxicillin and metronidazole were added in the amount equivalent to MIC.

A growth inhibition rate greater than 94% was observed with plain liposomes and nanocapsules (without drug). This showed that both the lipids as well as the two polymers used in preparation of the nanocapsules had antimicrobial activity. This was also clear from the agglutination assay and adherence study results. Phospholipids form a hydrophobic layer against the bacterium that does not allow it to attach to the mucosal surfaces. The "plug and seal effect" of the mucoadhesive polymers as well as the lipids checked the growth in placebo formulations also.

Both amoxicillin and metronidazole gave better and higher eradication rates under *in vitro* conditions as compared to *in vivo* conditions. The main reason for this might be the short residence time of the drugs in the gastric mucosa of animals and the possible degradation of drugs in the acidic conditions in the stomach. It was clear from the results of the *in vivo* bacterial clearance study that, unlike the plain drug, the formulations release the drug slowly, thus preventing entire drug from being exposed to the acidic media, thereby increasing its efficacy. Attachment of *H. pylori* to gastric mucin also depends on gastric pH.[37] Furthermore, it has been shown that the blank nanocapsules without drugs were also inhibitory *in vitro*, almost as good as the plain drugs; they might be effective *in vivo* also. They also inhibited the adherence of *H. pylori* to the gastric epithelial surface as described by *in situ* adherence assay. So, the other reason could be that the nanocapsules might have competed with the bacteria attached to the gastric mucosa and caused the partial flushing away of them that they combined with the activity of the drug gave an increased reduction in bacterial count. To explore this possibility, we treated *H. pylori* infected mice with the mixture of plain drug solution in the highest dose and blank nanocapsules, and reduction in mean bacterial count as well as clearance rate were

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determined. The clearance rate in this case was similar (60%) to that observed with plain drug solutions at the same dose level, and the reduction in mean colony count was also not statistically significant ($P < 0.05$). This clearly confirmed the hypothesis that encapsulation of drugs in the nanocapsules is necessary to protect the drug in the acidic environment and also to increase their retention in the stomach for prolonged duration, which gives the highest efficacy in eradication of *H. pylori* infection.

The method described by Nagahara et al.[6] was used to assess the *in vivo* usefulness of amoxicillin and metronidazole bearing mucoadhesive nanocapsules for the eradication of *H. pylori* formulations. Male Balb/c mice were used for the *in vivo* bacterial clearance study because the male model gives better colonization as compared to the female model.[38] Further, the Balb/c mouse is considered as an efficient model for *in vivo* studies of *H. pylori* infections. This model helps in elucidating *H. pylori* induced neoplasia and also evaluating the virulence factors in *H. pylori* infections. The mouse model is a short-term model and more economical, and mice are easy to handle and house. The model helps to study pathogenesis and immune response. The only drawback with the mouse model is that it cannot be used to study gastric cancers. C57BL/6 or Balb/c mice are preferred, as they have better colonizing ability and less intense inflammation.[39]

In order to improve the efficacy of the anti *H. pylori* agents, localized delivery in the stomach was proposed.[5] The use of acid stable antibiotics such as metronidazole and amoxicillin could increase the stomach specific delivery, increase gastric residence time, decrease diffusional distance, and allow more antibiotics to reach the infected site.[40]

5. Conclusion

Localized or topical delivery of drugs to the gastric lumen has been found as a useful tool in the treatment of *H. pylori* infections. In present study, we have tried to develop a mucoadhesive drug delivery system that could efficiently deliver antibiotics (amoxicillin and metronidazole) to the gastric mucosal region, which is the ecological niche of the spiral shaped Gram-negative bacteria. The use of liposome as the core protects the cell from damage, and the polymeric coating prevents back-diffusion of acid and also gives the plug and seal effect. The results of the *in vitro* bacterial cell−system interaction study have clearly shown that the combination of two drugs chosen has been found to be effective in *H. pylori* eradication. The results obtained also indicated the efficiency of the system to localize the drugs in the gastric environment. Based on the performance evaluation, it was clear that the prepared nanocapsules could be efficiently used to curtail the drug leaching of both hydrophilic and lipophilic drugs. The system was found to be rigid and tough for protecting the drug against the environmental conditions *in vivo*, which is clear from the *in vivo* clearance study. However, the toxicity issues due to the cationic nature of the polymer used for coating need to be considered before scaling up the system for clinical trials.

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