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Original Article

Eudragit S-100 Encapsulated Chitosan Coated Liposomes Containing Prednisolone for Colon Targeting: *In vitro*, *Ex vivo* and *In vivo* Evaluation

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ABSTRACT

Objective: The present study was carried out to design an efficient formulation to target the drug loaded liposomes exclusively to the inflamed colonic region by protecting the payload from releasing in the upper gastro intestinal tract (GIT) by using two polymers. **Methods:** Liposomes were prepared by lipid film hydration method followed by chitosan coating and finally encapsulation with eudragit S-100. The coated as well as uncoated liposomes were characterized for entrapment efficiency, size and surface charge. *In vitro* drug release study was carried out using three step pH gradient method. Acetic acid was used to induce colitis. *Ex vivo* tissue drug entrapment study was done using excised tissues of male albino rats. *In vivo* efficiency study was done by comparatively studying the histopathology and myeloperoxidase (MPO) activity. **Results:** Formulations showed entrapment efficiency (93-91%) and size (99-290nm). Formulation showed T_{lag} of 6 hrs and by the end of 16th h, 82% drug release was achieved. *Ex vivo* studies revealed higher tissue-drug entrapment (22.58±2.52%) in inflamed colon when compared to healthy colon (22.58±2.52%). Histopathological studies showed marked reduction in inflammation in case of ECLs (eudragit encapsulated chitosan coated liposomes) treated groups than standard drug treated rats. The reduction in healing was further confirmed by the MPO assay which showed significant reduction in MPO (900.25±1.31 ng/ml) levels of ECL treated groups than standard drug treated (1990.32±2.11 ng/ml) and IBD group (2125.54±1.56 ng/ml). **Conclusion:** The ECLs showed site specific release of liposomes and increased accumulation of liposome entrapped drug in the inflamed colon.

Key words: Acetic acid induced rat colitis, Colon targeting, *Ex vivo* tissue entrapment, Histopathological studies, IBD, MPO activity.

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INTRODUCTION

Targeting drug to the colon is desired in case of several pathological conditions. The ailments may range in severity from mild constipation and diarrhoea to more serious inflammatory bowel disease (IBD) like ulcerative colitis (UC) and Crohn's disease (CD) which may further progress to cancer. Several orally administered drugs that can act locally are either absorbed or deactivated in the upper GIT. The drugs that are absorbed may further produce unwanted systemic effects. Hence oral dosage forms that deliver high concentrations of the drug to the colon instead of upper GIT are desirable to treat colonic ailments.[1,2]

Prednisolone is a corticosteroid used in IBD to control symptoms and inducing remission in both UC and CD. Apart from the pharmacological actions, the drug has multitude of adverse systemic reactions following upper GI absorption.[3] Hence prednisolone was selected as a drug candidate for colon targeting. The present study is aimed at formulating prednisolone loaded liposomes coated with a biodegradable polysaccharide chitosan and then encapsulated within a pH sensitive eudragit S-100 shell. *In vitro* drug release study will be followed by *ex vivo* tissue-drug entrapment determination and *in vivo* efficiency study of the formulation. *In vivo* study that is conducted to determine the efficiency of the colon targeted formulation and compare it with standard prednisolone given orally is done using acetic acid induced rat colitis model. The efficiency is evaluated by microscopic examination of stained colonic tissue sections as well as determination of inflammatory marker enzyme MPO.

MATERIALS AND METHODS

Materials

Prednisolone B.P sample was gifted by Tianjin Tianyao Pharmaceuticals Ltd, China. Soy Phosphatidyl Choline (SPC) was gifted by LIPOID, Germany. Cholesterol was purchased from Lobachemie, India. Chitosan (Himedia labs) and Eudragit S-100 (Lobachemie) were used as polymers for coating the liposomes. Sodium taurocholate was procured from lobachemie and β-glucosidase and horse radish peroxidase from Himedia labs. All other reagents and solvents used were of analytical grade.

Approval from animal ethical committee

All procedures of the animal experimentation were approved by Institutional ethical committee of NGSM Institute of Pharmaceutical Sciences, NITTE deemed to be university, Karnataka, India under approval No. NGSMIPS/IAEC/MARCH-2018/87.

Animals

Male albino wistar rats of around 3 months old and weighing between 250-300 g were selected. The animals were procured from the animal house of NGSM Institute of Pharmaceutical Sciences, Mangalore, Karnataka, India. The animals were housed in cages kept in a room maintained at 25±2°C with alternating 12 h light and dark conditions. Rats were given RO water and commercial rat feed. Not more than 3 rats were housed in one cage.

METHODS

Preparation of liposomes

Lipid film hydration method was used to prepare liposomes.[4] Prednisolone (10 mg), SPC (60 mg) and cholesterol (30 mg) were dissolved in 10 ml chloroform. The clear solution obtained was dried on the inner walls of an 800 ml round bottom evaporating flask of a rotary evaporator at 50-55°C under vacuum. The lipid film was further dried by keeping the flask overnight in vacuum dessicator.[5] The dried film was then hydrated with 10 ml phosphate buffer pH 7.4 at 60-70°C and swirled for 15 min to form dispersion. The vesicles were downsized using a probe sonicator (Vibracell-sonics) for 5 min at 35% amplitude with cycles of 50 sec ON and 10 sec OFF. The size reduced vesicles were then stored at 4°C.[6]

Coating liposomes with Chitosan

A 2%w/v chitosan solution was prepared in 0.5 %v/v acetic acid by stirring for 24 h. Liposomal dispersion was slowly added to equal volume of chitosan solution drop wise under continuous magnetic stirring which was continued for 15 min after which the coating was allowed to stabilize by overnight refrigeration. Then the coated liposomes were purified by several cycles of centrifugation and washing with 0.5% v/v acetic acid to remove excess chitosan. Finally the coated liposomes were dispersed in deionized water and stored at 4°C until encapsulation process.[7]

Encapsulation of Chitosan coated liposomes within eudragit S-100 shell

A pH driven nano-precipitation method was done entirely in aqueous media thus achieving encapsulation without using any organic solvents that leave traces.[8] In, brief eudragit S-100 was solubilized in phosphate buffer pH 8. This alkaline polymer solution was mixed with equal volume of Chitosan coated liposome dispersion (solution A). This solution A was then added drop wise using a syringe into a beaker containing 0.25% v/v acetic acid solution (solution B) under magnetic stirring in a ratio of 1:9 of solution A and B respectively. The sudden drop in pH induces precipitation of eudragit around coated vesicles. Stirring was continued for 15 min followed by cycles of centrifugation at 10,000 rpm and washing with deionized water. The final pellet was redispersed in 10 ml deionized water and stored at 4°C until further analysis.

Particle size, zeta potential (ZP) and percentage entrapment efficiency (%EE) determination

Plain liposomes, Chitosan Coated Liposomes (CCLs) and Eudragit Encapsulated Chitosan Coated Liposomes (ECLs) were analyzed for size and ZP using Malvern-Nano S particle size analyzer which uses principle of dynamic light scattering and electrophoretic mobility of particles.[9,10]

To determine %EE, 1 ml aliquots of formulations were centrifuged at 15,000 rpm for 15 min at 4°C. The pellets were sonicated with 10 ml acetonitrile for 15 min to release the entrapped drug and then ultra centrifuged to separate the polymer and lipid residues from the drug in the supernatant. The supernatant was analyzed using HPLC (Shimadzu, Japan) and %EE was calculated using the equation,

$$\%EE = [C/C_d] \times 100$$

Where,

C = Concentration of drug in supernatant

C_d = Total drug in aliquot

In vitro drug release study

Drug release studies were performed by three step release method in three different simulated media.[11] Aliquots equivalent to 5 mg of prednisolone were placed in dialysis bag (Himedia) fastened to the paddle shaft of the dissolution apparatus and descended into 400 ml dissolution media maintained at 37±0.5°C. The paddle speed was set to 50 rpm. Drug release study was performed using HCL buffer pH 1.2 (0.2 M HCl and 0.2M KCl mixed buffer (gastric condition) for 2 h, phosphate buffer pH 7.4 containing model bile salt sodium taurocholate (small intestinal condition) for 3 h and phosphate buffer pH 6.8 (colonic condition) containing enzyme β-glucosidase for chitinolytic action for up to 11 h. Samples were withdrawn from the media at 30 min intervals for gastric conditions, at 1 h intervals for intestinal conditions and 2 h intervals for colonic conditions. Equal volume of fresh media was replenished after each sample withdrawal to maintain the sink conditions. The aliquots were filtered and analyzed for prednisolone concentration using HPLC.

Induction of colitis in rats and treatment

For experimentation, 30 rats were randomly divided into 5 groups: Healthy (no IBD, no treatment); Disease control (IBD, no treatment); Formulation treated (IBD, ECLs = equivalent to 5 mg/kg/day); Standard drug treated (IBD, drug solution = 5 mg/kg/day); Placebo (IBD, ECLs without drug). After an overnight fasting, the rats were lightly anaesthetized with ether and IBD was induced by rectal administration of 1 ml of 4 % acetic acid using an infant urinary catheter at 8 cm proximal to the anus for 30 s. To flush the colon, 1 ml of phosphate buffered saline (PBS) was similarly administered. The formulation, placebo and prednisolone was suspended in 1 ml saline and administered to the respective groups using gavages once daily from the fifth day after colitis induction and continued for 3 consecutive days.[12]

Sacrification and subsequent excision of tissues

Three days after drug treatment, the rats were sacrificed by high dose of ether inhalation. After sacrificing, a midline incision was made and stomach, a segment of small intestine and large intestine (about 3.5 cm) were quickly excised, washed with saline. The tissues were then divided as per the requirement of *ex vivo* study and histopathological analysis.

Ex vivo tissue-drug entrapment study

After sacrificing, stomach, small intestine and large intestine of healthy and disease control groups were washed gently with Krebs Ringer Bicarbonate (KRB) solution (sodium chloride 118 mmol/L, sodium bicarbonate 25 mmol/L, potassium dihydrogenphosphate 1.19 mmol/L, calcium chloride 1.03 mmol/L, magnesium sulfate 1.2 mmol/L, glucose 5 mmol/L albumin 1%; pH = 7.4) and everted using a glass rod inserted through the lumen. One end of the colon was tied to a needle and the opposite end was tied securely.[13] Continuous drug release method was employed by changing the pH of the mucosal media. Initially, the everted stomach was cannulated with the help of a needle and other end was ligated. The sac was filled with 2 ml of KRB solution was poured through the hypodermic needle.[14] The sac was lowered in to 100 ml simulated gastric media pH 1.2 with aeration at 37°C. In absorption studies, air was bubbled into the intestinal to obtain intestinal peristaltic movement. At the end of 2 h, the stomach sac was removed and the contents were transferred to everted small intestine sac treated in similar manner as the stomach. 33.3 ml of tribasic sodium phosphate was added to all the outer media and the pH was adjusted to 6.8 (3 h) along with sodium taurocholate. The sac was lowered into this media and process was continued. At the end of 5th h, the small intestine content was transferred to everted colon sac. One end was probed with a needle and other. 2 M NaOH was added to the outer media and pH was adjusted to 7.2.[15] β-glucosidase was also added to colonic media for chitinolytic activity. The colon sac was lowered into the media and process was continued for up to 11 h as *in vitro* drug release study. After each phase, the organ segments were removed from the incubation medium. The contents of the sacs were filtered in a 0.22 mm pore membrane separately. The concentration of prednisolone in the medium was determined by HPLC. The tissues were homogenized using 5ml acetonitrile and the content was centrifuged at 3000 RPM for 15 min the entire content was filtered using 0.45µ

membrane filter and the obtained solution volume was made up to 10 ml with acetonitrile. Samples were analysed by HPLC.

Histopathological study

The colon was fixed in 10% formalin in phosphate buffered saline for 1 week after which it was washed under running tap water for 2 h. The samples were then dehydrated in graded ethanol and then embedded in paraffin wax. Sections were taken using a microtome. Sections were placed on a glass slide and paraffin was removed with xylene, stained with haematoxylin-eosin. Tissues were examined under microscope for ulceration, inflammation and goblet cell structure.

MPO activity assay

Tissue specimens accurately weighed in range 200–400 mg were homogenized using 1 ml hexadecyl trimethyl ammonium bromide buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6) in ice cold conditions. After homogenization, the container was rinsed twice with 1 ml of HTAB. The pooled 3 ml homogenate of each specimen was sonicated and freeze-thawed three times to release the enzyme from the cells. The homogenate was centrifuged at 3,000 rpm for 5 min. 100 µl of supernatant was combined with 2.9 ml of 50 mM phosphate buffer, pH 6, containing 0.167 mg/ml of o-dianisidine dihydrochloride (ODA) and 0.0005% H_2O_2 in an ice bath. The absorbance is measured at 460 nm (Merck Spectroquant Prove-600 UV-Visible spectrophotometer) against the blank (i.e., reagent without the supernatant). The MPO levels were determined from standard plot constructed using standard horse radish peroxidase.[16]

Statistical analysis

The data of *ex vivo* studies and MPO activity assay were reported as mean ± standard deviation. Comparison among groups was done by performing unpaired t-test using Graphpad Prism software. Results having p value <0.05 were reported as significant.

RESULTS

Particle Size, Zeta Potential (ZP) and Percentage Entrapment Efficiency (%EE)

The average size, ZP and %EE data are given in Table 1. The results were compared between plain liposomes, chitosan coated (CCLs) and eudragit encapsulated chitosan coated liposomes (ECLs). The average particle size of plain liposomes was found to be 99.90±0.2 nm. After chitosan coating, the size increased to 235.8±0.1 nm. Further increase in size of 290.5±0.2 nm was observed after encapsulating the coated liposomes within eudragit S-100 shell. Zeta potential of liposomes was -33.1±0.3 mV which increased to +35.3±0.4 mV. After eudragit encapsulation, the charge reduced to -32.5±0.2 mV. Entrapment efficiency of plain liposomes was 94.29±0.5% which reduced slightly after chitosan coating (%EE=92.89±0.1%) and eudragit encapsulation (%EE=90.43±0.5%).

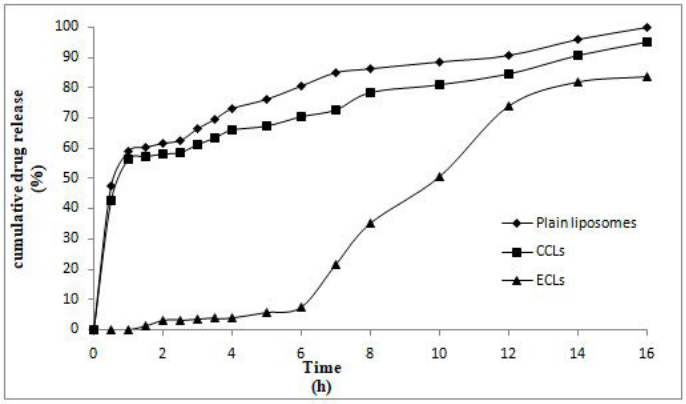

Figure 1: Cumulative drug release profiles of plain liposomes (♦), Chitosan coated liposomes (■) showing burst release within 1 h and Eudragit encapsulated chitosan coated liposomes (▲) showing Tlag of 5-6 h [Source: Original Data].

In vitro drug release profile

Plain liposomes and CCLs showed burst release of 47% and 42% respectively within 1 h in gastric media. Appreciable lag time of 5-6 h was observed with the release of more than 20% and maximum release of 82% drug by 16th h. The drug release profiles are shown in Figure 1.

Ex vivo tissue-drug entrapment study

The study was performed to evaluate the tendency of the drug to get entrapped within the inflamed tissue when incorporated in nanoformulations. The drug entrapped in inflamed colon tissue was found to be 22.58±2.52% which was highest when compared to healthy. The results are given in Table 2.

Histopathological examination

The efficiency of the formulation (ECLs) to deliver prednisolone to the inflamed colon in order to effectively treat the inflammation locally was evaluated by conducting histopathological examination after treatment. The formulation ECLs was successful in delivering the drug and localizing the therapy. The IBD induced rats treated with ECLs showed marked reduction in inflammation with evident remucosalization, reduction in focal ulceration and goblet cell distortion. The micrographs of the tissues are shown in Figure 2.

MPO activity assay

Estimation of MPO levels is a vital tool to estimate the extent of increase or reduction in inflammation of a tissue. The MPO levels of ECLs treated colon tissues were reduced significantly i.e. 900.25±1.31 ng/ml when compared to healthy and control groups. The data is reported in Table 3.

DISCUSSION

The negative charge, as well as desired particle size (around 99-100 nm) of uncoated liposomes, was due to the optimal cholesterol content that

Table 1: Particle size, zeta potential (ZP) and percentage entrapment efficiency (%EE) of plain liposomes, CCLs and ECLs (mean±SD) [Source: Original Data].

Formulation code	Particle size (nm)	ZP (mV)	EE (%)
Liposomes	99.90±0.2	-33.1±0.3	94.29±0.5
CCLs	235.8±0.1	+35.3±0.4	92.89±0.1
ECLs	290.5±0.2	-32.5±0.2	90.43±0.5

CCLs – Chitosan coated liposomes; **ECLs** – Eudragit encapsulated chitosan coated liposomes; **ZP** – Zeta potential; **EE** – Entrapment efficiency; **nm** – nanometers; **mV** – milli volts.

Table 2: Percentage entrapment of drug in healthy and disease control (IBD) tissues (mean±SD) [Source: Original Data].

Tissue Group	Tissue-drug entrapment in various tissues (%)			
	Stomach	Small intestine	Colon	External media
Healthy	0	1.17±0.75	9.42±2.68	72.56±2.67
Disease control	0	5.74±1.26	22.58±2.52	55.53±4.053

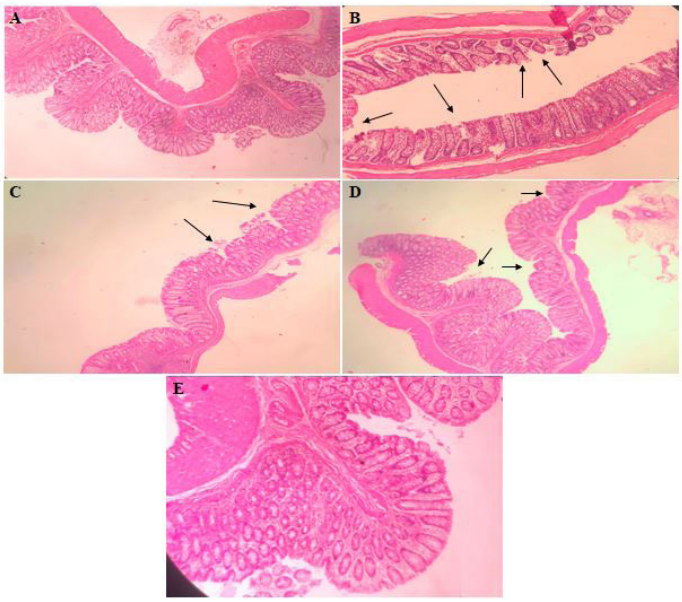

Figure 2: (a) Healthy rat colon with normal goblet cell structure and intact mucosa; (b)IBD induced colon with focal ulcerations, mucosal disruption and distorted goblet cell structure; (c)Placebo administered IBD induced colon with similar histopathological characteristics as the IBD induced group; (d) Standard drug treated colon showing slight signs of reduced ulcerations with slight goblet cell distortion; (e) ECLs treated colon with marked reduction in focal ulceration, intact goblet cell structure and evidence of re-mucosalization. [Source: Original Data]

Table 3: MPO levels in colon samples of different groups of rats (mean±SD) [Source: Original Data].

Groups	MPO Levels (ng/ml)
Healthy	150.56±2.21
Disease control (IBD)	2125.54±1.56
IBD+Placebo	2130.46±1.38
IBD+Std drug	1990.32±2.11
IBD+ECLs	900.25±1.31

MPO – Myeloperoxidase; **IBD** – Inflammatory bowel disease; **ng** – Nanogram.

imparted the charge and prevented aggregation due to the presence of interparticular repulsive forces. Addition of chitosan coat increased the particle size as well as imparted a higher positive charge on the particle surface due to positively charged functional groups of chitosan. Eudragit encapsulation again increased the size but imparted a negative charge on the CCLs surface. Entrapment efficiency was higher due to the lipid solubility and incorporation of the drug in the lipid phase of the liposomes. Exposure of drug loaded liposomes to successive coating process slightly reduced the encapsulation efficiency due to the mechanical stress of the processes.

Drug release profiles of plain liposomes and CCLs showed burst release of the drug since uncoated liposomes are unable to withstand the acidic and enzymatic gastric environment and also chitosan being soluble in acidic medium fails to protect vesicles. In case of plain liposomes 47% of the drug released in 30 min and for CCLs it was 42%. More than 70% of drug was released in the small intestine media before reaching the colonic region indicating the failure of both the systems for colon delivery of the drug. ECLs having eudragit concentration of 2% w/v for the stages where during 2 h in gastric conditions, 3 h in small intestine conditions showed only 4-5% drug release indicating that the liposomes are intact within the eudragit particles. Importantly, the formulation was resistant to degradation by sodium taurocholate. Eudragit shell solubilization occurred within 30 min of contact with colonic media which consequently led to drug release of more than 20%. Exposure to the colon, corresponding to 12 h on the drug release curve led to approximately 73% drug release, showing that most the liposomes would have been exposed at this point of time and therefore chitosan degradation and subsequent drug release would follow. At the end of 16th h, 82% drug release was achieved. The formulation showed a satisfactory lag time (T_{lag}) of 5-6 h which is required for colon targeted drug delivery systems.[17]

Nanoformulations have a tendency to adhere to inflamed colonic mucosa when compared to healthy tissue. To test this, everted sac method was used to evaluate the effect of ECLs on the entrapment of drug in both healthy and inflamed tissues as compared to pure drug. The percent drug entrapped for inflamed and healthy colon is 22.58±2.52 and 9.42±2.68 respectively. The difference was statistically significant with p-value <0.0001 at 95% confidence interval. The concentration of drug remaining outside the tissue i.e. in the media was also determined which represents the unentrapped drug and it was found to be 55.53±4.053 and 72.56±2.67 for inflamed and healthy colon respectively. This shows that the entrapment of formulation is much more to inflamed colon as compared to healthy.[18-20]

Histopathological features in acute on chronic colitis are mucosal erosions, crypt shortening, oedema and infiltration of neutrophils in the mucosa and lamina propria.[21] Compared to the normal group (Figure 2a), the IBD group exhibited marked erosion of the lamina propria, mucosa, focal ulcerations, distortion of goblet cells and inflammatory cell infiltration (Figure 2b). In standard drug-treated group (Figure 2d) and ECLs formulation-treated group (Figure 2e), mucosal erosion, distortion of goblet cells, ulceration and inflammatory cell infiltration tended to be less severe than those in the IBD group. Compared to the standard drug treated group, ECLs treated group showed faster healing and repair as there were very less ulcerations, mucosa showed signs of repair (re-mucosalization) and goblet cells distortion was also reduced, whereas in standard drug treated group, mucosa was not intact and prominent focal ulceration still persisted.

MPO is present in primary granules of neutrophils. In IBD, there is a larger infiltration of neutrophils in the inflamed tissue which leads to MPO release.[22] This is evident from the data given in Table 3 that there is significant increase (p-value<0.0001) in MPO activity of disease control IBD group (2125.54±1.56 ng/ml) as compared to the healthy group (150.56±2.21 ng/ml). Decrease in the activity of MPO in tissue is the sign of tissue repair and healing. It can be seen from the histogram a significant reduction in MPO level (p-value<0.0001) when treated with formulation ECLs (900.25±1.31 ng/ml) as that with standard prednisolone given orally (1990.32±2.11 ng/ml) and in contrast to IBD group.[23] The difference between the MPO levels of standard drug treated group and IBD control group was not statistically significant with p-value equal to 0.0651 (p>0.05). The colon targeted formulation ECLs inhibited MPO activity suggesting that infiltration of neutrophils thus proving its efficiency.

CONCLUSION

Liposomes have been reported to show selective accumulation at the inflamed sites. The studies conducted showed that the eudragit encapsulated chitosan coated liposome formulations (ECLs) having desired characteristics were successfully formulated by lipid film hydration method. Entrapment efficiency data showed successful incorporation of prednisolone within the liposomes. The dual polymer coating

protected the drug from releasing in the upper GIT by exhibiting an *in vitro* lag time of 5-6 h. *Ex vivo* studies showed that nanoformulations have a higher affinity towards inflamed tissues which further aids the efficiency of the formulation. The efficiency of the coated liposomes *in vivo* was confirmed by the results of histopathological studies and MPO activity assay of the colon tissue sections. Thus it can be concluded that such type of liposomal carrier system can be utilized to target the inflamed colonic tissue specifically.

However, there is a need to further establish the efficicacy of these formulations in human subjects.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

CCLs: Chitosan coated liposomes; **ECLs:** Eudragit encapsulated chitosan coated liposomes; **ZP:** Zeta potential; **EE:** Entrapment efficiency; **nm:** nanometers; **mV:** milli volts; **MPO:** Myeloperoxidase; **IBD:** Inflammatory bowel disease; **ng:** Nanogram.

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