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DEVELOPMENT AND INVESTIGATION OF EUDRAGIT S-100 ENCAPSULATED CHITOSAN COATED LIPOSOMES OF PREDNISOLONE FOR COLON TARGETING

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ABSTRACT: The objective of the study was to formulate coated liposomes containing Prednisolone for colon targeting and localization of the therapy to the inflamed colon mucosa. Liposomes were prepared using thin film hydration method. Liposomal formulations were optimized using 3^2 factorial design using varying concentrations of SPC and cholesterol as independent variables and % EE as the dependent variable. Optimized formulation F5 (60:30) was selected for successive coating with chitosan and eudragit S-100. Chitosan concentration of 1% w/v was taken as optimal based on ZP values. The coated liposomes were encapsulated in eudragit S-100 shell using a solvent-free method. The size if liposomes increased upon addition of the coatings. Fluorescent microscopy aided in clear visualization of chitosan coating around the vesicles. SEM images showed a transition surface appearance in the order smooth-rough-smooth for F5 and the coated liposomes. Eudragit S-100 coated liposomes were subjected to *in-vitro* drug release studies using simulated fluids for 16 h. ECL5 with 2% w/v eudragit showed sufficient lag time of 6 h. The formulation was able to protect the liposomes in gastric as well as small intestinal conditions, *i.e*., up to 5 h. Drug release of ECL5 followed zero order kinetics and fitted to Hixon-Crowell release model. The findings of the study conducted indicate a promising approach of the coated liposomes in the targeted release of the drugs.

INTRODUCTION: Colon targeting of a drug is an essential tool for the delivery of agents that get degraded in the upper GIT like proteins and peptides, drugs that produce adverse effects upon upper GI absorption, as well as drugs that are meant to be released in the colon for either systemic absorption or to treat local ailments like inflammatory bowel disease (IBD), amoebiasis, colon cancer and several other pathologies of colon [1].

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Several studies have been conducted by using pH controlled [2]; bacterial degradation controlled [3], time-dependent systems and also by utilizing a combination of those mechanisms [4, 5]. Liposomes are spherical lipid vesicles containing one or more lipid bilayers called lamellae which surround a central aqueous core [6].

Depending upon the solubility of the drug in the lipid or aqueous system, it can be incorporated in either of the two compartments. Liposomes are not usually meant for oral delivery due to their instability in upper GIT [7]. To target liposomes to the colon, they have to be encapsulated within a series of coatings that protect the vesicles in the stomach as well as the small intestine.

Here biodegradable polymers can be used as coating materials that resist degradation due to acid, bile salt and other digestive enzymes of the upper GIT that would dissolve the vesicles [8, 9, 10]. Having these problems, studies have also proved the interaction of liposomes with the mucosa thus exploiting this property for retaining the drug in the colonic mucosa [11].

Prednisolone is a steroid medication used in the treatment of IBD (Crohn's disease and ulcerative colitis). Upper GI absorption of Prednisolone may cause adverse effects like bloating, nausea, mild stomach ache, weight gain, adrenal insufficiency, menstrual period changes, and blood pressure variations [12]. To minimize these effects release of the drug in upper GIT has to be bypassed and high local drug concentration should be made available in the colon for a direct topical anti-inflammatory action on the inflamed mucosa [13].

The present work aims at formulating coated liposomes containing Prednisolone meant for colon targeting by using a combination of pH-dependent as well as bacterial degradation mechanisms for drug release followed by their characterization and evaluation.

MATERIALS AND METHODS:

Materials: Prednisolone B.P. sample was gifted by Tianjin Tianyao Pharmaceuticals Ltd., China. Soy lecithin (SPC) was gifted by LIPOID, Germany. Cholesterol was purchased from Loba Chemie, India. Chitosan (Himedia labs) and eudragit S-100 (Loba Chemie) were used as polymers for coating the liposomes. All other chemicals used were of analytical grade.

Experimental Design for Prednisolone Loaded Liposomes using 3^2 Full Factorial Design: Concentrations of SPC (X_1) and cholesterol (X_2) were independent variables and evaluated at three levels and experiment was conducted for all nine combinations. Percentage entrapment efficiency (% EE) was the dependent variable (Y). The variables are given in **Table 1**. Design Expert software version 11 was used to generate the batch and evaluate the design [14, 15].

TABLE 1: 3^2 FULL FACTORIAL DESIGN CONTAINING FACTORS, LEVELS, AND RESPONSE

Factors (Independent variables)	Levels		
	Low (-1)	Medium (0)	High (+1)
Amount of SPC (mg) X_1	30	60	90
Amount of cholesterol (mg) X_2	15	30	45
Response (Dependent variable)		Y = % EE	

Preparation of Liposomes: Liposomes were prepared by lipid film hydration method [16]. Prednisolone, SPC, and cholesterol were dissolved in 10 ml chloroform. The solution was dried on the inner walls of an 800 ml round bottom flask of a rotary evaporator at 50-55 °C under vacuum. Then the flask was kept overnight in a desiccator for further drying of the film [17]. The dried film was then hydrated with 10 ml phosphate buffer pH 7.4 warmed to 60-70 °C and agitated for 15 min to form a heterogeneous system of vesicles in the medium [18]. Finally, the vesicles were sonicated using a probe sonicator (Vibracell-sonics) for 5 min at 35% amplitude with cycles of 50sec ON and 10sec OFF [19]. The vesicles were then refrigerated at 4 °C.

Determination of % EE of Liposomes: 1 ml of liposome dispersion was centrifuged at 15,000 rpm using a refrigerated ultracentrifuge (REMI) at 4 °C for 10 min 20. The pellet containing the entrapped drug was lysed with acetonitrile using a bath sonicator for 10 min to release the drug, centrifuged again at 15,000 rpm for 10 min and the supernatant was analyzed for the entrapped (C) drug using HPLC (Shimadzu UFLC). Total drug (C_d) in the aliquot was measured by lysing the 1 ml of dispersion with acetonitrile followed by centrifugation and analyzing the drug content in the supernatant. Percentage entrapment efficiency was calculated using the equation,

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

Statistical Analysis and Optimization: Analysis and optimization of the formulation were done using response surface modeling (RSM) using Design expert (Version 11) software. Several statistical parameters such as coefficient of variance (CV), the coefficient of determination (R^2), adjusted R^2 and predicted the residual sum of squares (PRESS) were obtained from the software and compared to obtain the best fitting model.

Level of significance P<0.05 was considered. ANOVA was performed to determine the significance. Response surface plots were also derived to predict the effect of two factors on the response at a time. Numerical and graphical optimization methods were used to arrive at the optimal formulation by setting constraints.

Preparation of Chitosan-Coated Liposomes (CCLs): Chitosan was dissolved in 0.5% v/v acetic acid. The liposomal dispersion was slowly added to chitosan solution dropwise under magnetic stirring for 15 min then refrigerated overnight for stabilization of the chitosan coat. Then the CCLs were subjected to several cycles of centrifugation at 6000 rpm and washing with 0.5% v/v acetic acid to remove excess chitosan. Finally, the CCLs were dispersed in distilled water and stored at 4 °C [21].

Encapsulation of Chitosan-Coated Liposomes within Eudragit S-100 Shell: A pH driven nano-precipitation method reported by De Leo *et al*., was used done entirely in aqueous media thus achieving encapsulation without the use of any organic solvent [22]. Eudragit S-100 was dissolved in phosphate buffer pH 8. This alkaline polymer solution was mixed with an equal volume of CCLs dispersion. This solution was then added dropwise using a syringe into a beaker containing 0.25% v/v acetic acid solution under magnetic stirring in a ratio of 1:9 respectively. The sudden drop in pH precipitates eudragit around CCLs. Stirring was continued for 15 min followed by two cycles of centrifugation at 6000 rpm and washing with distilled water. The final pellet was redispersed in 10 ml distilled water.

Particle Size, Polydispersity Index (PDI) Zeta potential (ZP) and % EE Determination: CCLs and ECLs were analyzed for size and ZP using Malvern-Nano S particle size analyzer which uses the principle of dynamic light scattering and electrophoretic mobility of particles [23]. For % EE, 1 ml aliquots of both the coated liposomes were centrifuged at 15,000 rpm for 15 min at 4 °C. The pellets were lysed by sonicating with 10 ml acetonitrile for 15 min followed by centrifugation to separate the polymer and lipid residue from the free released drug in the supernatant. The supernatant was analyzed for drug concentration using HPLC and %EE was calculated using equation 1.

Fluorescence Imaging to Observe Chitosan Coating: CCLs were prepared by the method reported earlier with sodium fluoresce in solubilized in the chitosan solution. After coating, the excess dye was removed by repeated centrifugation and washing the pellet with distilled water. The dispersion was placed on a glass slide and sealed with a coverslip and observed using Leica DMC 4500 fluorescence microscope using 1000X magnification [24].

Surface Morphology by SEM Imaging: Uncoated liposomes were freeze-dried for 48 h for SEM Imaging. CCLs and ECLs were dried at 40 °C in a hot air oven for 48 h. The residues were further dried in a desiccator for 24 h. The specimens were scattered on adhesive pads and mounted on specimen mounts, sputter coated with palladium and then examined with the electron microscope (Zeiss Gemini) [25].

***In-vitro* Drug Release Study and Determination of Release Kinetics:** Drug release studies were carried out in simulated GI conditions at three different GI conditions [26]. Aliquots equivalent to 5 mg of Prednisolone were placed in dialysis bag 27 tied to the paddle shaft of the dissolution apparatus and lowered into 400 ml of release media maintained at 37 ± 0.5 °C. The stirring speed was set to 50 rpm.

Drug release study was performed using simulated gastric fluid (SGF) pH 1.2 (0.2 M HCl and KCl mixed buffer) for 2 h, simulated intestinal fluid (SGF= phosphate buffer pH 7.4) containing model bile salt sodium taurocholate for 3 h and simulated colon fluid (SCF = phosphate buffer pH 6.8) containing β-glucosidase for chitinolytic action for 11 h. 1 ml samples were taken at 30 min intervals for gastric conditions, at 1 h intervals for intestinal conditions and 2 h intervals for colonic conditions. An equal volume of fresh medium was replaced after each sampling to maintain the sink conditions. The aliquots were filtered and analyzed using HPLC. Drug release kinetics was determined by applying the *in-vitro* release data to various release models such as zero-order, first-order, Korsmeyer-Peppas, Higuchi and Hixson Crowell.

HPLC Analysis: HPLC equipped with a PDA and UV-Visible detector (Shimadzu-UFLC, Kyoto,

Japan) and a C18 column was used. The mobile phase was acetonitrile and water (50:50). Sample injection volume was 10 µl. The column temperature was maintained at 25 °C. Samples for % EE determination were prepared in acetonitrile which helped precipitate out the phospholipids and other polymer residues.

For this purpose, the calibration curve of Prednisolone was constructed using acetonitrile. For drug release studies, calibration curves were constructed in all the three release media. The linearity equations were used to calculate the drug concentration.

RESULTS AND DISCUSSION:

% EE, Size, PDI and ZP of Liposomes: The % EE size data of Prednisolone loaded liposomes is given in **Table 2**. The %EE of liposomes varied from 30 to 95%. Significant increase in % EE was observed with increase in cholesterol level but entrapment reduced with the further increase of cholesterol which may be attributed to increasing in rigidity and packing of the membrane and vesicles thus not providing sufficient space for the drug molecules to remain trapped in the lipid layers which in turn reduces the % EE [28].

Increase in %EE with SPC concentration can also be observed which may be because a greater amount of SPC provides additional space for drug molecules to accommodate [29]. The mean size of liposomes was in the range of 99 to 278 nm. Lower cholesterol levels made the lipid layers flexible to allow the entry of water into the vesicles causing an increase in size. As cholesterol level increases, the lipid membrane becomes more lipophilic which hinders the entry of water inside the vesicle resulting in smaller size [30]. The surface charge of liposomes was found to be in the range of -20 to -33 mV due to the negative phospholipid groups and will also aid the electrostatic attraction of the positively charged chitosan onto its surface [31].

TABLE 2: %EE, SIZE, ZP AND PDI OF PREDNISOLONE LOADED LIPOSOMES

Formulation Code	SPC (mg) X_1	Cholesterol (mg) X_2	% EE	Size (nm)	ZP (mV)	PDI
F1 (-1,-1)	30	15	35.60 ± 0.9	252.4 ± 0.1	-24.9 ± 0.9	0.210 ± 0.1
F2 (-1,0)	30	30	47.73 ± 0.8	210.2 ± 0.2	-30.7 ± 0.2	0.292 ± 0.2
F3 (-1,+1)	30	45	31.73 ± 0.7	230.5 ± 0.5	-20.9 ± 0.1	0.214 ± 0.1
F4 (0,-1)	60	15	76.62 ± 0.8	140.2 ± 0.2	-26.3 ± 0.8	0.251 ± 0.2
F5 (0,0)	60	30	94.29 ± 0.5	99.90 ± 0.2	-33.1 ± 0.3	0.182 ± 0.1
F6 (0,+1)	60	45	70.67 ± 0.8	164.5 ± 0.1	-24.3 ± 0.2	0.316 ± 0.2
F7 (+1,-1)	90	15	50.78 ± 0.6	271.4 ± 0.3	-28.6 ± 0.2	0.382 ± 0.1
F8 (+1,0)	90	30	65.46 ± 0.9	251.3 ± 0.5	-31.8 ± 0.4	0.391 ± 0.0
F9 (+1,+1)	90	45	43.86 ± 0.6	278.5 ± 0.2	-22.1 ± 0.1	0.323 ± 0.2

* Mean ± SD, n = 3.

Optimization of Liposomal Formulation: Quadratic polynomial equation was obtained as,

$$(\%\ EE)\ Y = +92.33 + 7.5\ X_1 - 2.83X_2 - 0.75\ X_1\ X_2 - 34.5\ X_1^2 - 17.5_2^2$$

ANOVA data given in **Table 3** shows the model F-value of 155.05 implies the model is significant. P-values <0.05 indicate X_1, X_2, X_1X_2, X_1^2 and X_2^2 were model significant terms. The predicted R^2 0.9618 agreed with adjusted R^2 0.9897; *i.e.*, the difference is less than 0.2.

By setting goals on the dependent variable, desirability, and contour plots were obtained as **Fig. 1** and **2** respectively for optimization. From the contour plots, the predicted formulation (60:28) was found to be matching F5 (60:30) having a similar response (93% EE). The desirability value for F5 was found to be 0.64 (nearing to 1). The response surface curve was also obtained to predict the effects of factors on the response as given in **Fig. 3**.

TABLE 3: ANOVA AND R^2 DATA

Parameter	Sum of Squares	df	Mean Square	F-value	P-value		R^2	Adjusted R^2	Predicted R^2
% EE (Y)	3380.92	5	676.18	155.05	0.0008	significant	0.9961	0.9897	0.9618

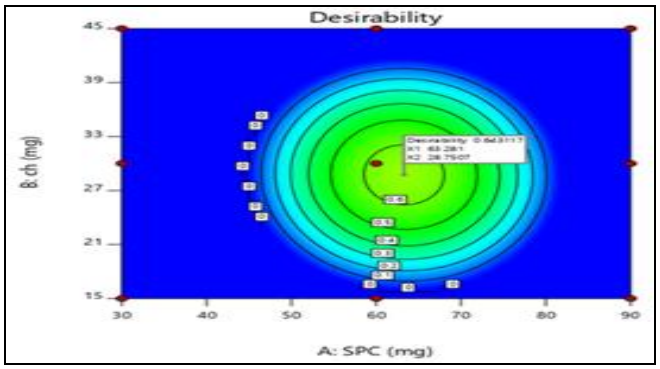

FIG. 1: DESIRABILITY PLOT

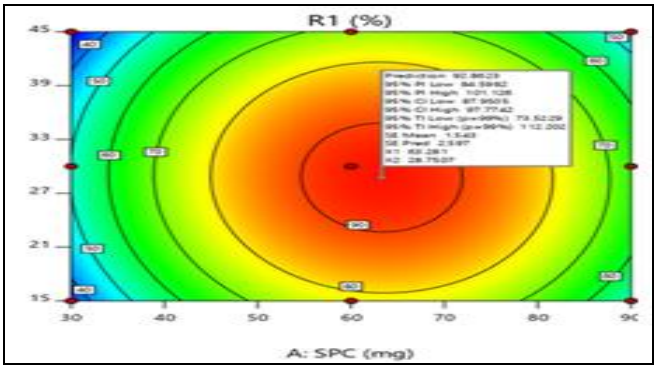

FIG. 2: CONTOUR PLOT

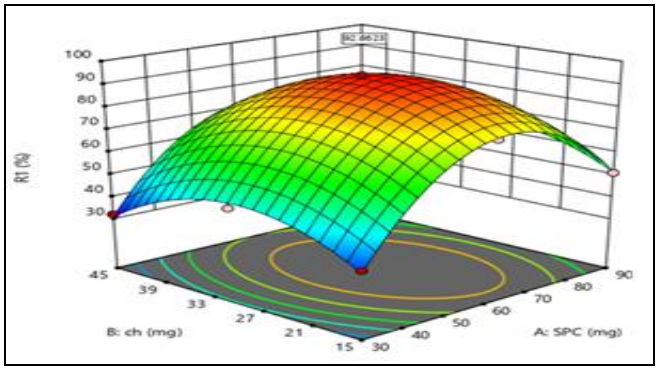

FIG. 3: RESPONSE SURFACE PLOT FOR AMOUNT OF SPC (X_1) AND CHOLESTEROL (X_2) ON % EE (Y)

Characterization of CCLs and ECLs: Coating with the polymers showed an increase from the original size (99.9 nm) of the liposomes. The PDI values of 0.2 to 0.4 indicate the presence of particles of the diverse size range. This may be due to agglomeration or breakage of particles during the coating process. The ZP of CCLs escalated from -27 to + 35 mV due to chitosan molecules as the concentration of chitosan increased from 0.1 to 1% w/v [32]. Further increase in concentration up to 3% showed no change in charge. This plateau phase as shown in **Fig. 4** was taken as a parameter to fix chitosan concentration to 1% w/v (CCL4) assuming that the liposome surface has been saturated with the polymer and further increase in concentration was unnecessary.

The ZP of ECLs decreased with increase in concentration attaining a negative charge due to the presence of uncapped end carboxyl groups on the polymer chain extremities of eudragit.

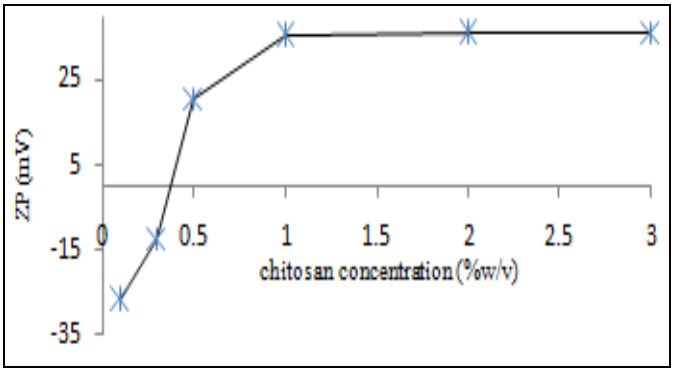

FIG. 4: EFFECT OF CHITOSAN CONCENTRATION ON ZP

TABLE 4: SIZE, ZP, PDI, AND % EE OF CCLs AND ECLs

Formulation	Size (nm)	PDI	ZP (mV)	%EE
CCL1 (0.1% w/v Chitosan)	120.2 ± 0.3	0.315 ± 0.1	-27.2 ± 0.3	94.01 ± 0.2
CCL2 (0.3% w/v)	147.1 ± 0.2	0.286 ± 0.2	-12.5 ± 0.2	93.78 ± 0.1
CCL3 (0.5% w/v)	175.2 ± 0.2	0.322 ± 0.1	+20.8 ± 0.3	93.25 ± 0.3
CCL4 (1 % w/v)	235.8 ± 0.1	0.248 ± 0.1	+35.3 ± 0.4	92.89 ± 0.1
CCL5 (2% w/v)	249.2 ± 0.4	0.295 ± 0.2	+35.9 ± 0.2	91.95 ± 0.4
CCL6 (3 % w/v)	260.0 ± 0.5	0.367 ± 0.3	+36.1 ± 0.1	91.37 ± 0.2
ECL1 (0.25% w/v Eudragit S-100)	251.4 ± 0.4	0.285 ± 0.3	+15.1 ± 0.2	91.22 ± 0.4
ECL 2 (0.5% w/v)	256.2 ± 0.5	0.305 ± 0.2	-10.9 ± .01	91.10 ± 0.2
ECL 3 (1 % w/v)	263.8 ± 0.2	0.412 ± 0.2	-21.0 ± 0.2	90.59 ± 0.3
ECL 4 (2% w/v)	290.5 ± 0.2	0.311 ± 0.3	-32.5 ± 0.2	90.43 ± 0.5
ECL 5 (3 % w/v)	330.2 ± 0.1	0.273 ± 0.1	-35.9 ± 0.3	90.02 ± 0.2
ECL 6 (4 % w/v)	370.5 ± 0.3	0288 ± 0.2	-39.4 ± 0.2	88.91 ± 0.1

*n = 3, mean ± SD

The coated liposomes showed a slight reduction in % EE when compared to uncoated liposomes as shown in **Table 4** indicating that some amount of drug has leaked during the coating process.

Fluorescence Microscopy of CCL4: The coating could be seen as a distinct layer around the liposomes as shown in **Fig. 5**.

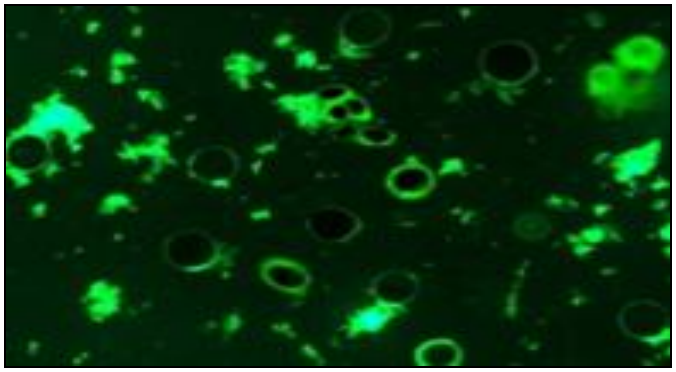

FIG. 5: FLUORESCENT MICROGRAPH OF CCLs

SEM Imaging: Liposomes shown in **Fig. 6a** were nearly spherical with slight deformity due to the drying process. CCLs appeared as clusters of spherical particles as seen in **Fig. 6b**. ECLs in **Fig. 6c** appeared to be larger compared to F5 and CCLs due coating. ECLs surface appeared smooth in contrast to that of rough CCLs.

***In-vitro* Drug Release Study:** Drug release profiles of F5 and CCL4 in **Fig. 7** show burst release of the drug since uncoated liposomes are unable to withstand the acidic and enzymatic gastric environment and chitosan being soluble in acidic medium fails as a protection for the vesicles. In case F5, 47% of the drug released in 30 min and for CCLs it was around 42%. More than 70% of the drug released in the small intestine media before reaching the colon indicating the failure of both the systems for colon delivery.

Drug release profiles of ECLs point out that gastroprotection and simultaneous drug release was dependent on polymer concentration. ECLs having lower eudragit concentrations (0.25 and 0.5% w/v) show only slight resistance in the gastric environment with drug releases of around 40% and 10% respectively at the end of 2 h. At the end of 5th h, 40 to 60% release was observed which was due to solubilization of eudragit coat in the simulated intestinal media with added sodium taurocholate. The lower eudragit concentrations delayed the drug release initially but soon allowed entry of the upper gastric and intestinal media to enter the vesicles and leak out the drug.

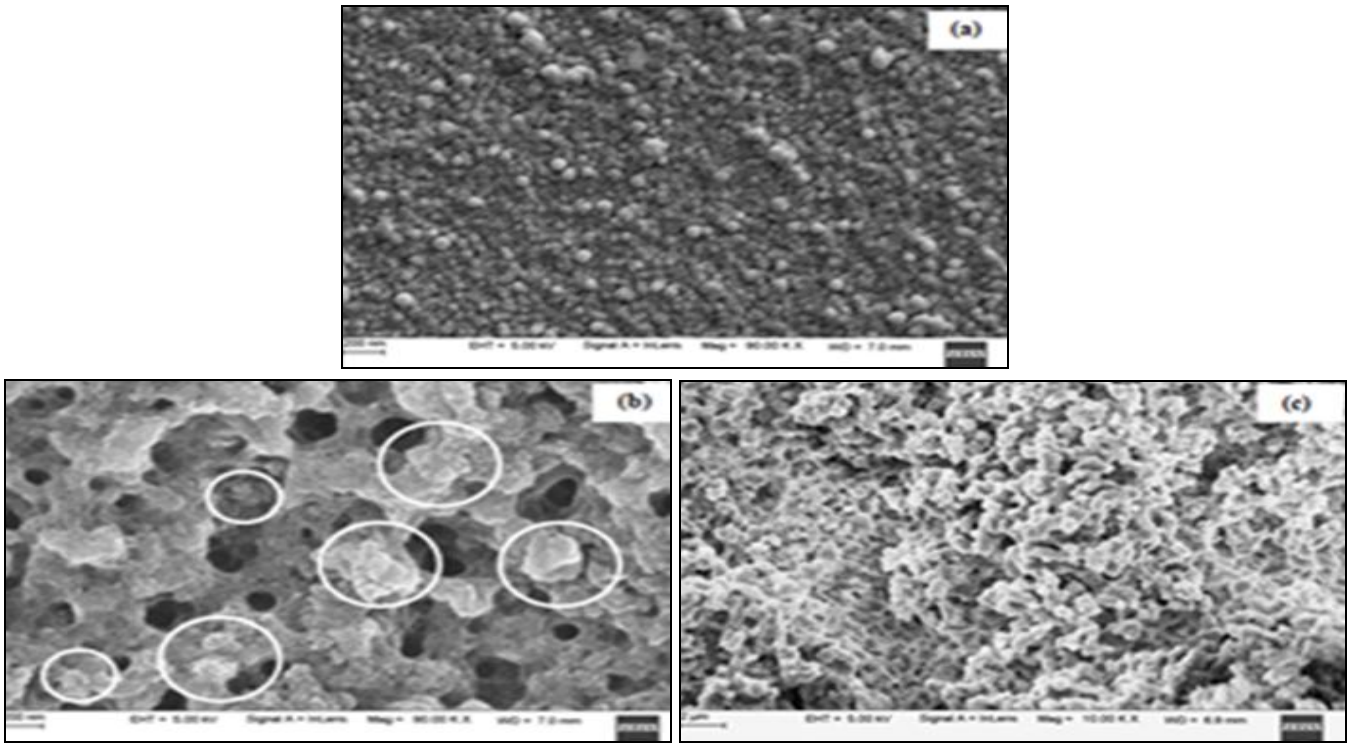

FIG. 6: SEM IMAGES OF (a) PLAIN LIPOSOMES, (b) CCLs AND (c) ECL

Further increase of polymer concentrations (1-2%) showed a higher degree of protection from the upper GI environment. Eudragit coating of 2% during 2 h in gastric conditions, 3 h in small

intestine conditions, showed very little drug release (around 4-5%) indicating that the liposomes are maintained within the eudragit shell. The formulation was resistant to degradation by sodium taurocholate. Once eudragit shell degradation occurs, (within 30 min of exposure to large intestine conditions) significant drug release (>20%) starts. Exposure to the large intestine conditions, corresponding to 12 h showed approximately 73% of drug release. This shows that most of the liposomes would have been exposed at this point and therefore chitosan solubilization and subsequent drug release can occur. By the end of 16th h, 82% drug was released. The formulation ECL5 showed a satisfactory lag time (T_{lag}) of 6 h which is required for colon targeted drug delivery systems [33].

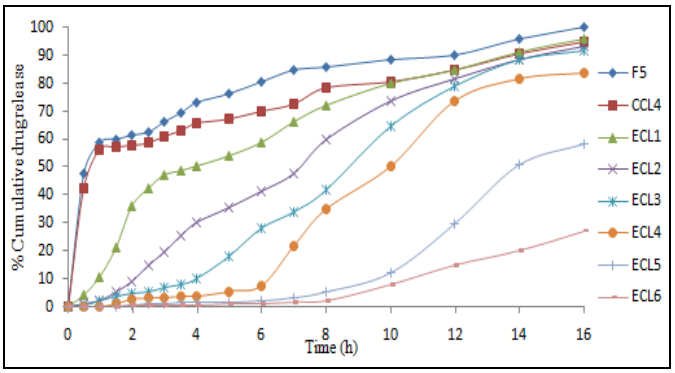

FIG. 7: DRUG RELEASE PROFILES F5, CCL4, AND ECLs

TABLE 5: DRUG RELEASE KINETICS

Formulation	Zero-order	First order	K- Peppas	Higuchi	Hixon-Crowell
	r^2	r^2	r^2	r^2	r^2
ECL1	0.871	0.975	0.970	0.969	0.980
ECL2	0.977	0.965	0.889	0.940	0.990
ECL3	0.971	0.908	0.747	0.845	0.942
ECL4	0.922	0.876	0.643	0.757	0.896
ECL5	0.794	0.749	0.465	0.591	0.765
ECL6	0.825	0.808	0.492	0.620	0.814

The drug release kinetics data in **Table 5** indicates that the drug release of ECL4 follows zero order kinetics with r^2 = 0.922. The data were then fitted to Hixon-Crowell equation to determine the release mechanism. The r^2 = 0.896 indicates a close to a linear pattern of drug release. On accounting all ECLs, they showed a linear zero order release kinetics and Hixon-Crowell cube root law. The law is applicable for drug release/dissolution from monodisperse [34]. The zero order kinetics seemed to be predominating to Hixon-Crowell cube root law.

CONCLUSION: The present work aimed at formulating colon targeted liposomes by coating first with chitosan and then encapsulating within eudragit S-100 polymer to deliver Prednisolone, a model drug to locally treat IBD. Formulations were evaluated for % EE, size, ZP and drug release in simulated GIT media. The entrapment efficiency of plain liposomes F5 was found to be 94%. Optimization of liposomes was done by 3^2 factorial design.

The predicted response (93% EE) was in agreement with the actual response of F5 which was selected for further studies. The particle size of liposomes was found to be between 99-280 nm, whereby an increase in size (up to 370 nm) post coating was observed. Chitosan coating concentration was optimized by using ZP as criteria. Formulation CCL4 (1% w/v) was taken as optimal since no further change in ZP was observed at chitosan concentration higher than 1% w/v. SEM images showed a transit from the smooth surface of liposomes to rough appearance after chitosan coating followed by smooth, even surface after eudragit encapsulation. Drug release studies were performed in simulated gastric conditions for 16 h. F5 and CCL4 were unstable in SGF. ECLs showed an increase in lag time with increased polymer concentration.

Formulation ECL4 (2% w/v) showed desired lag time (6h) with drug release less than 10%. The model bile salt did not show any signs of degradation in the SIF. Maximum drug release (82%) was observed after complete depletion of eudragit shell and solubilization of chitosan coat in the SCF in the presence of β-glucosidase. By drug release profiles obtained, formulation ECL4 having 2% eudragit S-100 and 1% chitosan coating was found to be the best candidate for targeted delivery of Prednisolone into the colon.

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CONFLICT OF INTEREST: There are no conflicts of interest.

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