## Supporting Material

# Folate Grafted Thiolated Chitosan Enveloped Nanoliposomes with Enhanced Oral Bioavailability and Anticancer Activity of Docetaxel

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## Methods

### Synthesis of Thiolated Chitosan (CS-TGA)

Thioglycolic acid was initially coupled with chitosan *via* EDAC coupling [1]. Briefly, 1 % chitosan solution was prepared in 1% acetic acid. To this solution, TGA (1%) and EDAC (50 mM) were added while stirring. Hydroxylamine (50 mM) was added to the reaction mixture to avoid oxidation during synthetic procedure. The pH was adjusted to 5.0 with 1M HCl solution and the reaction mixture was kept stirred for 4 h to produce thiolated chitosan (CS-TGA).

In order to eliminate unreacted materials and to obtain purified CS-TGA, the mixture was dialyzed under dark for 3 days in a dialyzing membrane (Cutoff value 12KDa), at 10 ºC: 1 time with 5 mM HCl solution, 2 times again with the same medium having 1 % NaCl to quench ionic interaction between cationic polymer and anionic sulfhydryl compound and finally 2 times with 1 mM HCl to adjust the pH at 4.0. Thereafter, the CS-TGA solution was lyophilized and stored at 4 $^{o}$C until further use.

### Synthesis of Folate grafted thiolated chitosan (FA-CS-TGA)

Folic acid (FA) was grafted to CS-TGA through EDAC coupling [2]. Briefly, 10 mg of FA and EDAC was dissolved in 5 mL DMSO and added to 1 % (m/v) CS-TGA solution in deionized water. The pH of reaction mixture was adjusted to 9.0 with 0.5 M NaOH and stirred for 16 h. Folate grafted thiolated chitosan (FA-CS-TGA) was purified *via* dialysis against PBS (pH 7.4) and deionized water, 3 days each. Purified FA-CS-TGA was freeze dried and stored at 4 °C until further use.

### Synthesis of NLs

All the NLs formulations were prepared by dry film rehydration technique [3, 4]. Briefly, 50 mg of lipid mixture containing choline, DPPC, oleic acid and cholesterol were dissolved in 5 mL solvent mixture containing chloroform and methanol (9:1, v/v). The organic solvent was evaporated under vacuum using rotary evaporator (Heidolph, Germany) to obtain a dried thin lipid film. The produced film was dried thoroughly in a vacuum chamber, rehydrated with PBS (pH 7.4) and incubated at 60 °C (above phase transition temperature) for 1 h with repeated vortexing to produce multi-lamellar vesicles. Liposomal suspension was sonicated using bath type sonicator (Elmasonic X-tra 70) for 10 min at 60 $^{o}$C to further reduce the size of liposomes. These empty NLs were used for *in-vitro* characterization of various parameters. DTX loaded NLs were produced in the same manner except that DTX was dissolved with lipids mixture in organic solvents, the remaining procedure being the same. Free drug was separated from drug loaded NLs by centrifugation at 4000 rpm for 5 min. Liposomes were freeze dried and stored at -20 $^{o}$C till used for further studies.

### Characterization of formulation

### Determination of thiol group content

Quantification of thiol group attached to the chitosan backbone was spectrophotometrically determined using Ellman's Reagent [5]. Briefly, 0.5 mg of each of CS, CS-TGA and FA-CS-TGA was hydrated in 250 µL of deionized water separately. To this suspension, 250 µL of phosphate buffer (pH 8.0, 0.5 M) and 500 µL of freshly prepared Ellman's reagent was added. The samples were incubated for 3 h at room temperature and supernatant was separated from the precipitated polymers through centrifugation. Thereafter, supernatant was transferred to a 96-well plate, and

the absorbance was measured at a wavelength of 430 nm with a microtitre-plate reader (PerkinElmer, USA). TGA standards were used to calculate the amount of thiol groups immobilized on the polymer.

**Formation of disulfide bonds**

Disulfide content was determined to quantify the total amount of free thiol groups present on both CS-TGA and FA-CS-TGA [6]. Briefly, 0.5 mg of polymer was hydrated in 350 µL of deionized water and to that 650 µL of phosphate buffer (pH 6.8, 0.05 M) was added. After 30 min, 1 mL of freshly prepared sodium borohydride solution (1 %, m/v) was added. The mixture was incubated for 1 h at 37 $^{o}$C. Afterwards, 200 µL of HCl (5 M) was added to decompose the remaining sodium borohydride. The solution was neutralized by the addition of 1 mL phosphate buffer (pH 8.5, 1 M) and 100 µL Ellman's Reagent (0.4 %, m/v) in phosphate buffer (pH 8.0) was immediately added. After 1 h of incubation, aliquot of 300 µL was transferred to the microplate and absorbance was measured at 430 nm using microtitre-plate reader (PerkinElmer, USA). The amount of free thiol group was determined by subtracting the calculated thiol groups in earlier step from the total thiol groups immobilized on the modified polymers.

**Encapsulation Efficiency**

The encapsulation efficiency of all the formulations developed was calculated by re-suspending 2 mg of lyophilized formulation in 2 mL of Tris-HCl buffer. The suspension was then sonicated for 30 min in water bath to rupture the particles and setting drug free in the water. To this mixture, 1mL of mobile phase (acetonitrile : methanol : buffer) was added and sonicated for another 15 min to dissolve the drug and extract further traces entrapped in the formulations. The solution was filtered through 0.22 µ syringe filter and transferred to HPLC vial [7]. The quantity of DTX loaded in 2 mg of formulation was estimated through the HPLC-PDA method newly developed for DTX using HPLC (waters e2695, USA) with isocratic mobile phase flow 0.8 mL/min at 230 nm. Same method was repeated in triplicate and average was calculated to determine encapsulation efficiency from all the formulations by using the formula:

$$Encapsulation\ Effeciency = \frac{Amount\ of\ drug\ in\ formulation}{total\ amount\ of\ drug} X\ 100 \quad [2]$$

***In-vitro* drug release studies**

The dialysis membrane diffusion technique was applied to study the *in-vitro* drug release from NLs and ENLs [8]. The weighed quantity of formulations containing drug equivalent to 5 mg was re-suspended in deionized water and placed in a dialysis membrane (Cutoff value 12 KDa) sealed and immersed in 30 mL of phosphate buffer (pH 7.4, 0.1 M) containing tween 80 (1% m/v) to maintain the sink conditions as DTX has less solubility in buffer solution. Pure DTX was used as a standard to compare the drug release from the formulations. The system was maintained at 37 ºC ± 0.5 and 100 rpm. Samples were collected at predefined intervals, filtered through 0.22 µ syringe filter and analyzed through HPLC (waters e2695, USA) using the same method developed to calculate encapsulation efficiency. The release data was then analyzed with DDSolver, a free Microsoft Excel Add-in, to study the release kinetics from NC's [9].

***Ex-vivo* permeation enhancement and efflux pump inhibition analysis**

*Ex-vivo* permeation enhancement was analyzed using everted sac method by comparing the synthesized formulations with DTX dispersion [10]. Briefly, the study was conducted on intestine of healthy rats weighing between 200-250 g and were used for the first time for experiment. The rats were anesthetized with chloroform and abdomen was opened with middle incision. The jejunum was immediately removed, thoroughly washed with Krebs ringer solution (pH 6.5) and was cut into pieces of 4-5 cm. The intestine was everted by carefully passing a narrow glass rod from one end of the intestine and then gently rolling it on a glass rod. All the pieces were stored in oxygenated Krebs ringer solution at 4 $^{o}$C till further use. 1% Tween 80 was added to enhance the wettability of DTX. Each segment was tied at one end with silk suture and 1 mL of sample (1 mg/mL) was carefully filled in the sac using hypodermic syringe and the other end was tied with silk suture. Verapamil (100 µg/mL), a P-gp inhibitor, was filled in one sac to compare the apparent permeation enhancement with NLs. All filled sacs were immersed in tubes filled with 10 mL of oxygenated Krebs Solution and incubated at 37 $^{o}$C under gentle mechanical shaking. The samples were collected

from the surrounding medium at definite time and replaced with the same amount of fresh solution. The same method was repeated to study basolateral to epical transport to study p-gp inhibition activity of ENLs. Non everted sections were filled with sample solutions and tied with silk. The samples obtained were analyzed using HPLC and apparent permeability was calculated using following equation:

$$Apparent\ Permeability\ (\mu g/cm2) = \frac{Concentration\ \times\ Volume}{Mucosal\ Surface\ Area} \quad [4]$$

Mucosal surface area was calculated by assuming intestine a cylinder and using formula:

$$Mucosal\ Surface\ Area\ (cm2) = Circumference\ (\pi \times diameter) \times Length \quad [5]$$

***In-vitro* cytotoxicity studies**

*In-vitro* cytotoxicity of pure DTX and all the formulations were screened through MTT assay using breast cancer (MD-MB-231) cell line [11]. Briefly, MB-231 cell line was seeded in 96-well optiplate at a density of 6000 cells per well in DMEM with 10 % FBS and incubated for 24 h in humidified incubator with 5 % $CO_2$. The cells were incubated with 5 µg, 2.5 µg, 1.25 µg, 0.625 µg, 0.312 µg, 0.156 µg, 0.05 µg and 0.001 µg DTX and formulations containing equivalent drug concentration and blank NLs and ENLs for 72 h. After incubation, the medium was replaced with fresh DMEM and 10 µL of MTT was added to each well and plate was incubated for another 4 h. After 4 h, MTT-containing media was aspired off and 100 µL of DMSO was added in each well to dissolve the formazan crystals formed by living cells. Then the absorbance was measured at 570 nm using multi plate reader (Perkin-Elmer, USA). Untreated cells with 100% viability were taken as control and the cells without addition of MTT were used as blank to calibrate the instrument. $IC_{50}$ values for each formulation was calculated using Graphpad Prism 6.02 software. The results were performed in triplicate and expressed as mean ± SD [12].

**Results and Discussion**

**Synthesis of thiolated chitosan (CS-TGA)**

Thiolated polymer was synthesized by modifying the chitosan (CS) backbone *via* covalent linkage with thioglycolic acid (TGA) through amide bond formation between amino moieties of chitosan and carboxylic acid groups of TGA. Quantification of the thiol groups, immobilized on thiolated chitosan (CS-TGA) revealed an average 845 ± 67 µM of thiol moieties per gram of the polymer. In addition, 128 ± 73 µM disulfide bonds and 596 ± 14 µM primary amino groups were present per gram of CS-TGA. The obtained lyophilized CS-TGA appeared as white, odorless powder of fibrous structure. The lyophilized polymer was stored at 4 $^oC$ and found stable towards oxidation throughout the course of the study. The FTIR spectra of CS and CS-TGA is shown in **Fig. S2.** The spectrum of CS clearly showed absorbance bands at 1654 $cm^{-1}$ (amide I), 1604 $cm^{-1}$ ($NH_2$) bending and 1382 $cm^{-1}$ (amide III). The band at 1156 $cm^{-1}$ (asymmetric stretching of COOOC bridge), 1072 $cm^{-1}$ and 1023 $cm^{-1}$ (skeletal vibration because of -COO stretching) are important features of its saccharin structure. However, in the CS-TGA spectrum peaks at 3351 $cm^1$ and 3209 $cm^{-1}$ represented O-H and N-H stretching and peak observed at 1630 $cm^{-1}$ was assigned to acylamino group. Also the intensity of peak around 1607 $cm^{-1}$ decreased, indicating that amino groups were partly conjugated to TGA [13].

**3.1.1. Synthesis of folic acid grafted CS-TGA (FA-CS-TGA)**

The grafting of folic acid with CS-TGA was based on the mechanism of carbodiimide chemistry. The carboxylic acid group of folic acid were activated by EDAC to generate an amine reactive O-acylisourea intermediate. Afterwards, this intermediate reacted with the free amino groups of thiolated chitosan to form folic acid conjugated (FA-CS-TGA) as shown in **Fig. S1.** The quantity of disulfide linkage and primary amino groups were found to be 158 ± 47 µM and 361 ± 22 µM per gram of polymer. The attachment of folic acid to CS-TGA was confirmed by FTIR spectroscopy and shown in **Fig. S2.** FTIR spectrum of folic acid grafted TCS showed characteristic peak at 3372 $cm^{-1}$ and 3274 $cm^{-1}$ corresponding to alcohol (O-H stretching) and primary and secondary amine (N-H stretching) respectively. The appearance of two characteristic peak at 1662 $cm^{-1}$ and 1585 $cm^{-1}$ representing carbonyl (C=O) stretching and NH associated (N-H) bending in secondary amine. In addition, a new sharp band at 1314 $cm^{-1}$ corresponded to C-N

stretching of secondary amine. Hence, all the data suggested that free $NH_2$ groups of CS-TGA were converted to NH groups [2].

**Table S1:** Coded values of independent factors and dependent responses for optimization of NLs Synthesis obtained from CCD using Design Expert Software.

| *STD* | *Run* | *Factor 1 A:DPPC Mg* | *Factor 2 B:Choline Mg* | *Factor 3 C: OA mg* | *Factor 4 Cholesterol7 Mg* | *Response1 Particle Size nm* | *Response 2 PDI* | *Response 3 EE %* | *Response 4 ZP eV* |
|---|---|---|---|---|---|---|---|---|---|
| 8 | **1** | -1.000 | -1.000 | -1.000 | -1.000 | 180 | 0.324 | 60.5 | 15.4 |
| 17 | 2 | 0.000 | 0.000 | 0.000 | 0.000 | 165 | 0.213 | 69.2 | 23.6 |
| 20 | 3 | 0.000 | 0.000 | 0.000 | 0.000 | 170 | 0.241 | 68.3 | 23.5 |
| 15 | 4 | 0.000 | 0.000 | -1.000 | 0.000 | 177 | 0.281 | 63.5 | 15.3 |
| 5 | 5 | -1.000 | 1.000 | 1.000 | -1.000 | 205 | 0.342 | 53.8 | 32.7 |
| 1 | 6 | 1.000 | 1.000 | -1.000 | 1.000 | 216 | 0.351 | 67.2 | 28.5 |
| 12 | 7 | 0.000 | 0.000 | 0.000 | 1.000 | 216 | 0.265 | 70.3 | 15.8 |
| 14 | 8 | 1.000 | 0.000 | 0.000 | 0.000 | 155 | 0.291 | 61.8 | 21.7 |
| 10 | 9 | 0.000 | 1.000 | 0.000 | 0.000 | 176 | 0.185 | 74.4 | 28.5 |
| 13 | 10 | -1.000 | 0.000 | 0.000 | 0.000 | 161 | 0.271 | 59.3 | 28.5 |
| 11 | 11 | 0.000 | 0.000 | 0.000 | -1.000 | 194 | 0.254 | 74 | 29.3 |
| 3 | 12 | 1.000 | 1.000 | 1.000 | -1.000 | 184 | 0.143 | 72.4 | 24.5 |
| 2 | 13 | -1.000 | 1.000 | -1.000 | 1.000 | 193 | 0.264 | 70 | 32.5 |
| 6 | 14 | 1.000 | -1.000 | -1.000 | -1.000 | 170 | 0.306 | 76.4 | 26.7 |
| 16 | 15 | 0.000 | 0.000 | 1.000 | 0.000 | 154 | 0.184 | 81.6 | 24.6 |
| 18 | 16 | 0.000 | 0.000 | 0.000 | 0.000 | 165 | 0.214 | 72.7 | 21.4 |
| 9 | 17 | 0.000 | -1.000 | 0.000 | 0.000 | 153 | 0.264 | 71.4 | 22.4 |
| 4 | 18 | -1.000 | -1.000 | 1.000 | 1.000 | 187 | 0.165 | 77.4 | 27.4 |
| 7 | 19 | 1.000 | -1.000 | 1.000 | 1.000 | 155 | 0.181 | 73.6 | 24.7 |
| 21 | 20 | 0.000 | 0.000 | 0.000 | 0.000 | 174 | 0.212 | 72.8 | 21.6 |
| 19 | 21 | 0.000 | 0.000 | 0.000 | 0.000 | 166 | 0.255 | 71.3 | 20.4 |

**Table S2:** Characterization of particle size, PDI, zeta potential and encapsulation efficiency of NLs and ENLs formulation synthesized. Results are shown as Mean ± S.D. of 3 different experiments.

| **Formulation** | **Particle size (nm)** | **Polydispersity Index (PDI)** | **Zeta potential (mV)** | **Encapsulation Efficiency (%)** |
|---|---|---|---|---|
| NLs | 132.50 ± 2.34 | 0.22 ± 0.01 | - 43.10 ± 0.34 | - |
| NLs-DTX | 246.50 ± 1.39 | 0.32 ± 0.05 | - 22.60± 0.18 | 71.49 ± 3.82 |
| ENLs-DTX | 328.50 ± 0.36 | 0.36 ± 0.01 | + 18.30 ± 2.52 | 83.47 ± 5.62 |

***Table S3:*** *Results of viscoelastic parameters i.e. storage modulus (G') and loss modulus (G'') and apparent viscosity of the thiolated chitosan (CS-TGA), Folate grafted thiolated chitosan (FA-CS-TGA), NLs and ENLs and their corresponding mucin (5%)/formulation mixtures*

| | Time | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1hr | | | 6hrs | | | 12hrs | | |
| **Formulation** | **G'(Pa)** | **G''(Pa)** | **V(Pa.S)** | **G'(Pa)** | **G''(Pa)** | **V(Pa.S)** | **G'(Pa)** | **G''(Pa)** | **V(Pa.S)** |
| CS-TGA | 18.31 ± 3.22 | 12.52 ± 4.18 | 0.08 ± 2.14 | 56.34 ± 4.56 | 42.67 ± 4.30 | 1.14 ± 1.38 | 113.44 ± 23.58 | 68.34 ± 7.52 | 3.53 ± 1.21 |
| CS-TGA with Mucin | 28.41 ± 4.25 | 17.35 ± 3.50 | 0.18 ± 2.45 | 69.27 ± 3.35 | 54.75 ± 5.40 | 3.33 ± 1.67 | 82.32 ± 8.31 | 77.64 ± 5.27 | 7.44 ± 1.43 |
| FA-CS-TGA | 13.45 ± 2.67 | 19.44 ± 2.87 | 0.04± 2.36 | 51.37 ± 5.13 | 42.10 ± 6.32 | 0.09 ± 1.45 | 105.50 ± 11.37 | 64.89 ± 8.76 | 2.23 ± 1.21 |
| FA-CS-TGA with Mucin | 20.44 ± 5.32 | 16.43 ± 3.64 | 0.35 ± 3.34 | 89.29 ± 7.55 | 65.21 ± 7.39 | 3.12 ± 1.57 | 195.43 ± 8.55 | 134.11 ± 13.45 | 5.83 ± 1.46 |
| NLs | 7.71 ± 4.47 | 6.95 ± 3.67 | 0.02 ± 1.62 | 29.76 ± 2.47 | 21.94 ± 3.27 | 0.06 ± 1.13 | 48.13 ± 7.45 | 38.51 ± 7.40 | 0.94 ± 1.26 |
| NLs with Mucin | 9.31 ± 6.65 | 8.41± 4.58 | 0.06 ± 1.44 | 37.40 ± 23.26 | 31.43 ± 27.57 | 0.15 ± 1.19 | 63.59 ± 15.38 | 57.25 ± 21.52 | 1.28 ± 1.15 |
| ENLs | 63.42 ± 6.44 | 57.35 ± 6.37 | 0.03 ± 1.36 | 95.42 ± 4.56 | 71.34 ± 7.89 | 0.07 ± 1.22 | 178.91 ± 12.40 | 161.44 ± 11.76 | 3.31 ± 1.10 |
| ENLs with Mucin | 80.43 ± 8.78 | 64.31 ± 4.33 | 0.45 ± 1.67 | 645.16 ± 65.87 | 479.44 ± 16 | 2.75 ± 1.45 | 3542.82 ± 47.64 | 2978.43 ± 54 | 17.93 ± 1.64 |

***Table S4:*** *Dissolution data modeling based on in-vitro drug release of various formulations to determine drug release mechanism from NLs and ENLs*

| Formulation | Zero Order | | Korsmeyer-peppas | | Higuchi | | Hixon-Crowell | |
|---|---|---|---|---|---|---|---|---|
| | $R^2$ | $K_o$ | $R^2$ | N | $R^2$ | $K_H$ | $R^2$ | $K_{HC}$ |
| DTX | 0.46 | 1.31 | 0.98 | 0.39 | 0.94 | 9.15 | 0.70 | 0.05 |
| NLs | 0.24 | 2.01 | 0.95 | 0.41 | 0.86 | 14.36 | 0.92 | 0.02 |
| ENLs | 0.58 | 1.65 | 0.98 | 0.43 | 0.96 | 11.40 | 0.88 | 0.01 |

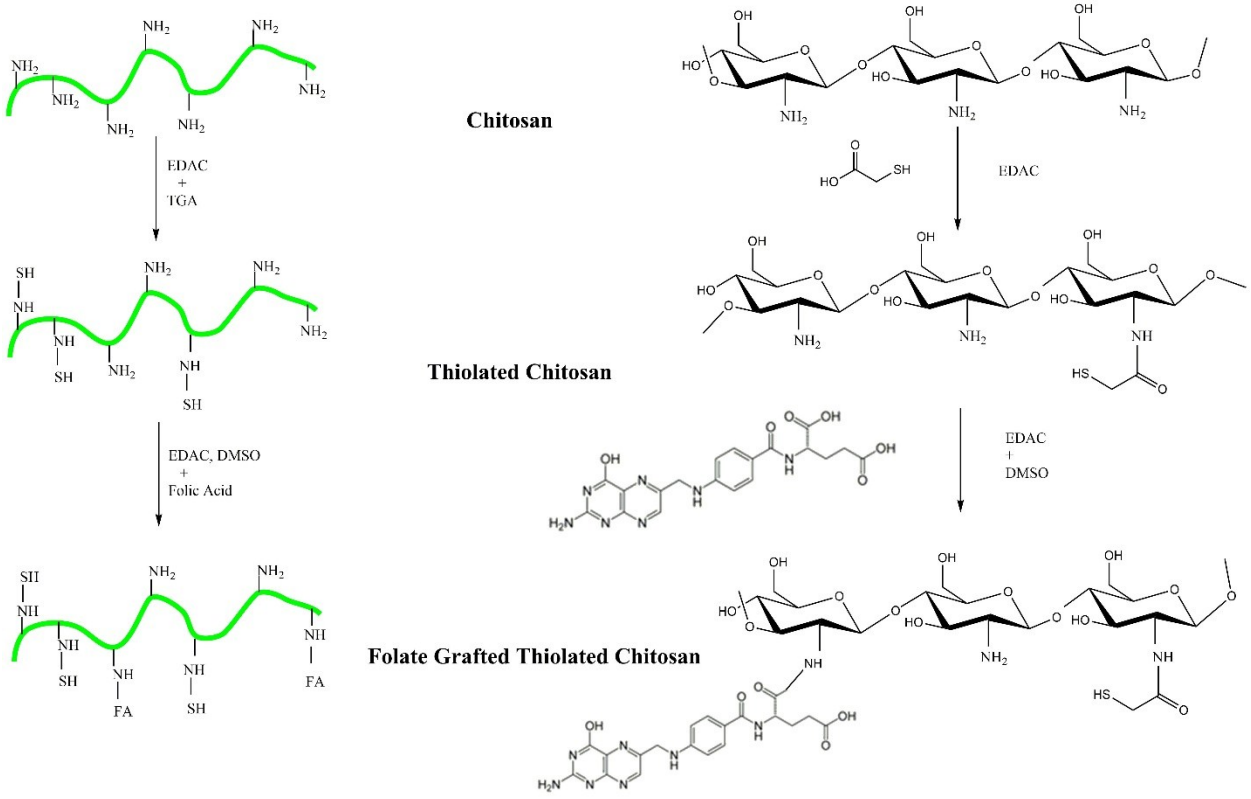


**Figure S1:** Synthetic pathway showing synthesis of Thiolated chitosan (CS-TGA) and Folate grafted thiolated chitosan (FA-CS-TGA).

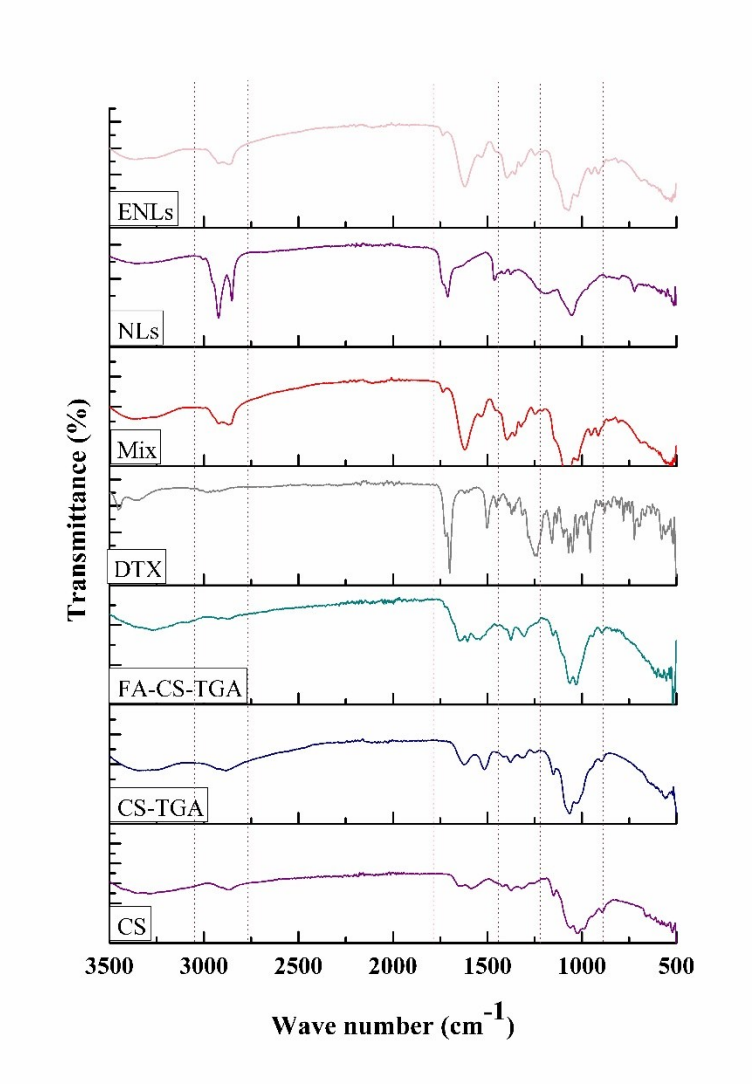


**Figure S2**: FTIR spectra of chitosan (CS), thiolated chitosan (CS-TGA), folate grafted thiolated chitosan (FA-CS-TGA), physical mixture (Mix), NLs and ENLs.

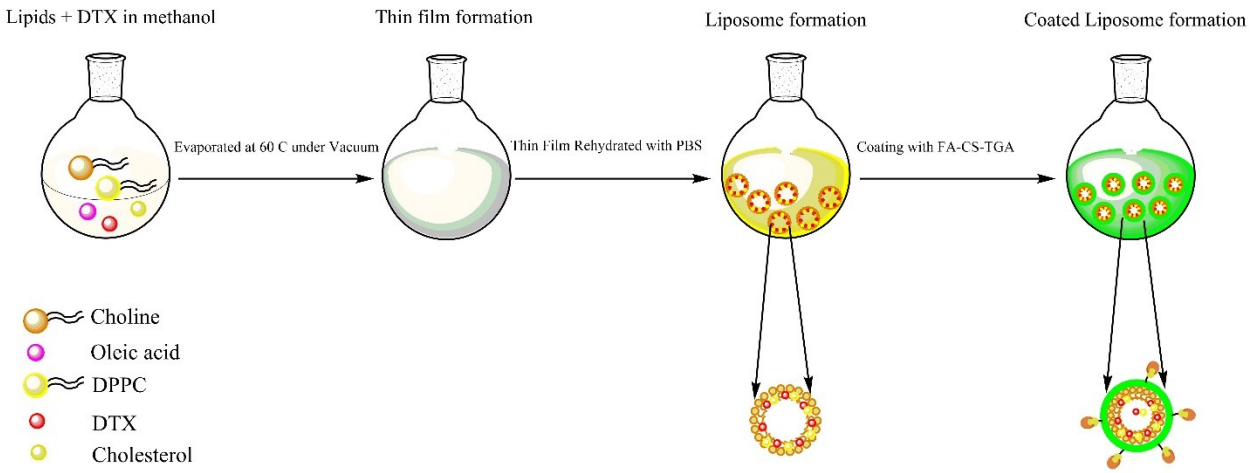


**Figure S3:** Schematic representation of the synthesis of NLs and ENLs

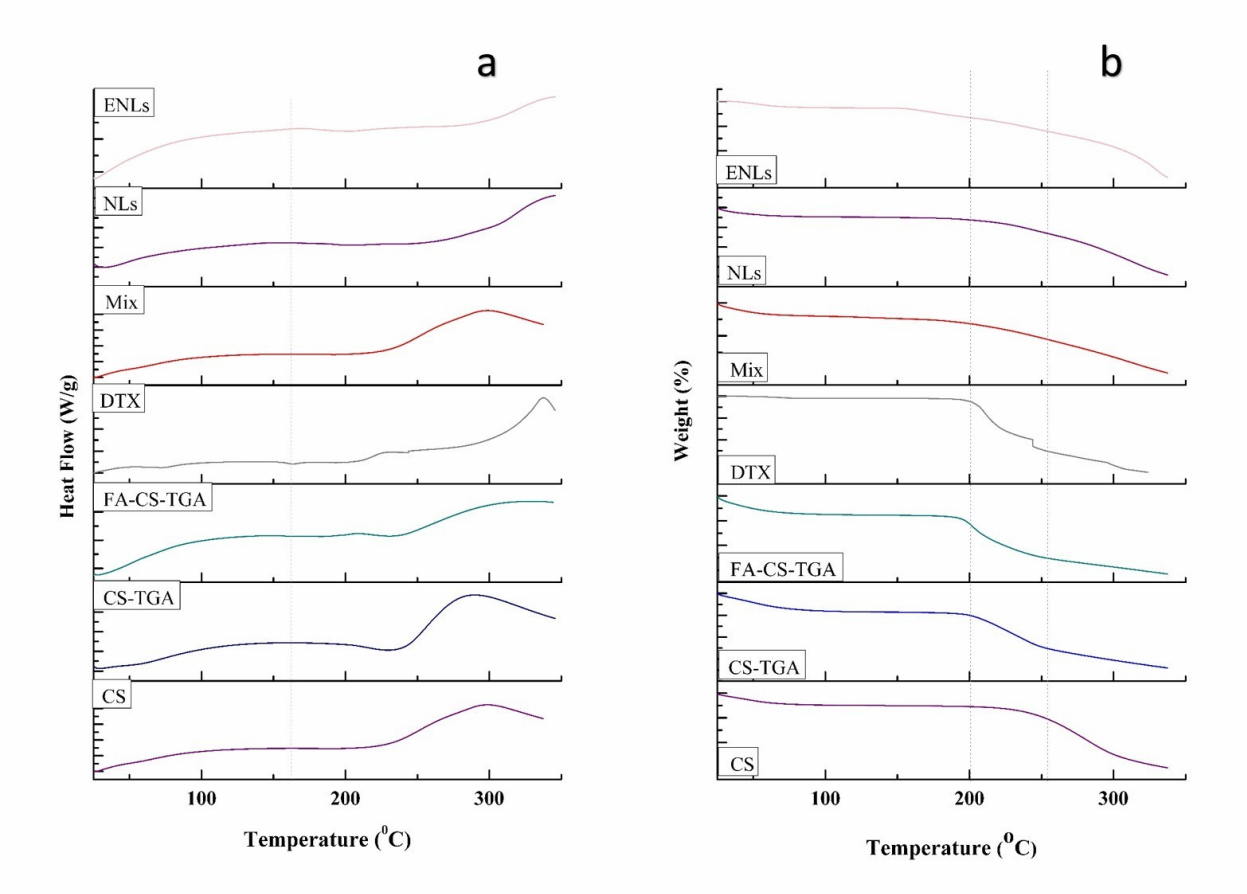


**Figure S4:** (a) DSC analysis and (b) TGA analysis of chitosan (CS), thiolated chitosan (CS-TGA), folated grafted thiolated chitosan (FA-CS-TGA), NLs and ENLs

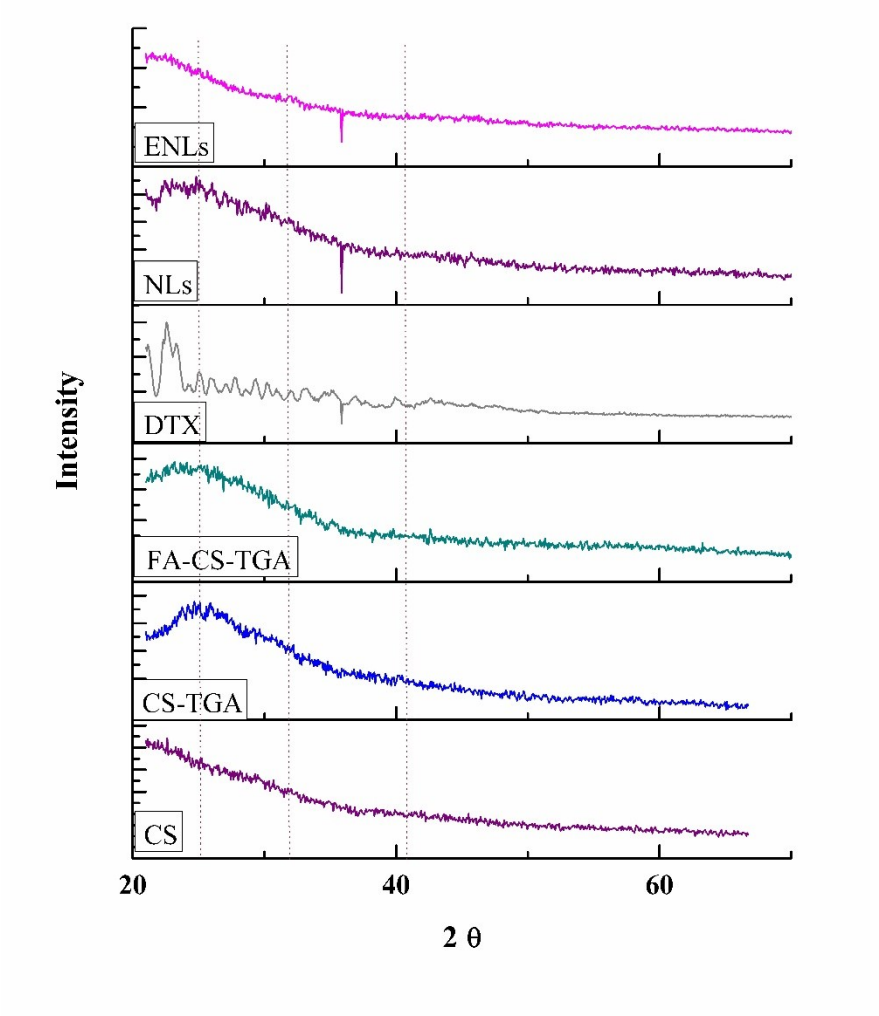


**Figure S5:** Powder X-ray diffraction studies (PXRD) of chitosan (CS), thiolated chitosan (CS-TGA), folate grafted thiolated chitosan (FA-CS-TGA), NLs and ENLs.

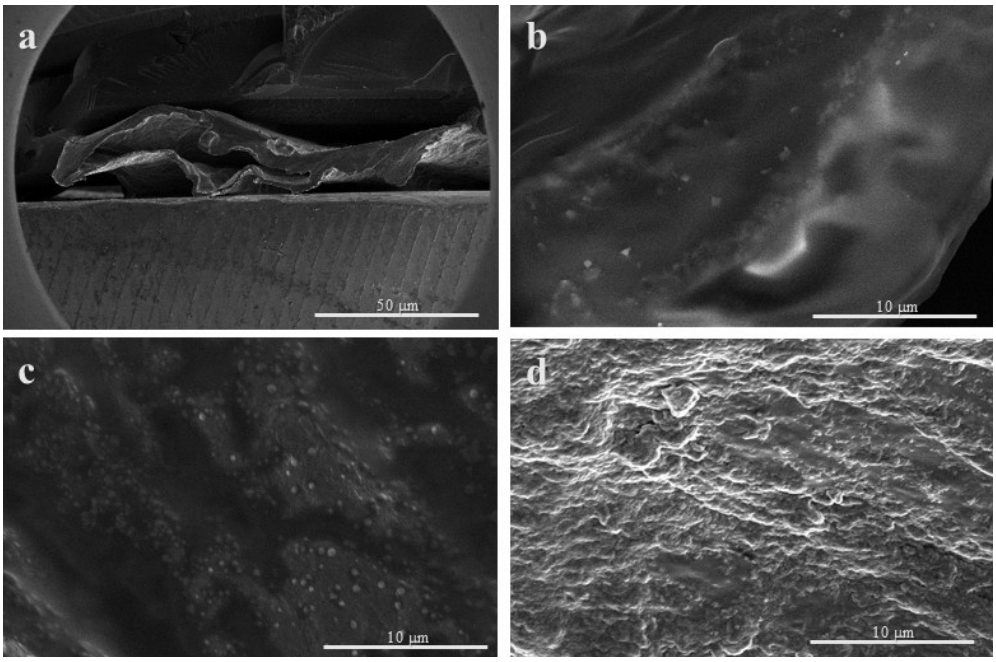


**Figure S6:** Scanning electron micrographs of rat intestine after permeation enhancement studies (a) Rat intestine (b) Transverse section (TS) of Rat intestine, (c) Basal surface of intestine and (d) Epical surface of intestine

## References


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