Experimental

This chapter describes different experimental techniques used for the synthesis of LDH based materials and their bioconjugation. In addition characterization techniques are also briefly portrayed.

2.1 Synthesis

2.1.1 Materials

Anti-cancer drug, Raloxifene hydrochloride (RH), and Poly(ε-caprolactone) (PCL; Mn ~80,000) were purchased from Sigma-Aldrich. $LiNO_3$, $Al(NO_3)_3 \cdot 9H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$,$NaNO_3$, Na_2CO_3, and Na_3PO_4 were purchased from Merck India Ltd. Salmon testes DNA was purchased from Sigma-Aldrich. The pGFP-p53 purchased from Addgene (Cambridge, MA, USA). Dulbecco's Modified Eagle Medium (DMEM), penicillin and streptomycin were also purchased from Sigma, USA. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), MTT), Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin and fetal bovine serum were obtained from Himedia, India. Acridine orange, ethidium bromide, rhodamine B and FITC were purchased from Sigma, USA. Dimethyl sulphoxide (DMSO) and paraformaldehyde were purchased from Merck India Ltd.

2.1.2 Synthesis of Mg-Al based layered double hydroxides

The Mg-Al based LDHs with varying interlayer anions were prepared by using coprecipitation technique [Markland et al., 2011]. At first, a mixture solution of 0.5 M $Mg(NO_3)_2 \cdot 6H_2O$ and 0.25 M $Al(NO_3)_3 \cdot 9H_2O$ with $[Mg^{2+}]/[Al^{3+}]$ molar ratio of 2:1 was prepared in 75 ml of deionized water. 100 ml of 1.5 M solution of Na_2CO_3, $NaNO_3$, and Na_3PO_4 were prepared separately and were placed in five-neck flat bottom flask. Five necks of the flask were fitted with one gas inlet-outlet, two burettes, one condenser and one with pH meter probe. Prepared solutions were then degassed by purging purified nitrogen gas. After that, the previously prepared mixture solution of $Mg(NO_3)_2 \cdot 6H_2O$ and $Al(NO_3)_3 \cdot 9H_2O$ was added drop wise into Na_2CO_3, $NaNO_3$, and Na_3PO_4 solutions separately and the solutions were stirred vigorously using a magnetic stirrer (rpm 600). 1 M NaOH solution, fitted in one burette, was added drop wisely to the above mentioned

three different solutions until the pH reached the value of 10. The whole solution was then stirred continuously at 60 ^{o}C for overnight. Appearance of white gelatinous precipitate indicated the formation of MgAl-LDHs (having three different anions intercalated LDHs, NO_3–LDH, CO_3–LDH, PO_4–LDH and were abbreviated as LN, LC and LP, respectively). The precipitates were recovered by centrifugation (6000 rpm) at room temperature and were washed thoroughly with ethanol and deionized water followed by drying in air oven at 60 ^{o}C for 48 h.

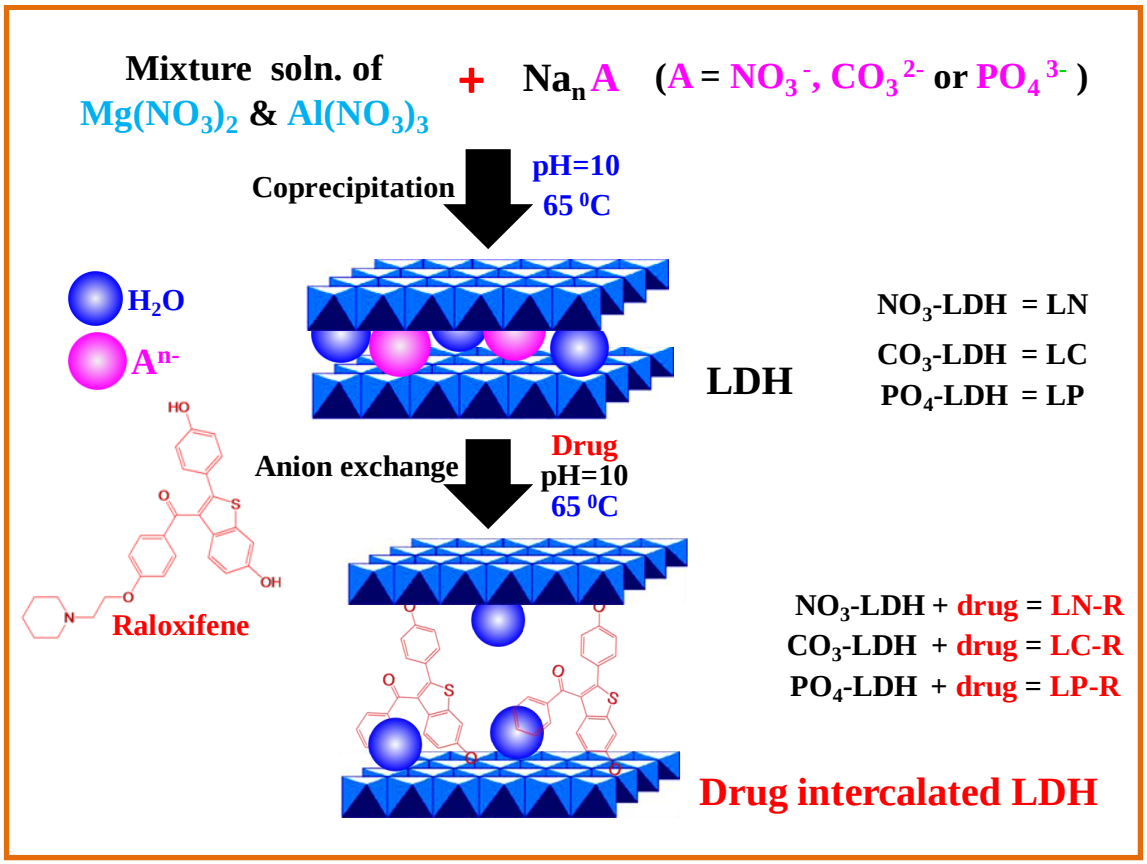

Figure 2.1: Reaction scheme showing synthesis of various LDHs with varying interlayer anions and intercalation of drug molecules into the interlayer gallery of LDH.

2.1.3 Preparation of anticancer drug intercalated Mg-Al LDHs

Anticancer drug (Raloxifene hydrochloride; RH) intercalated Mg-Al LDHs with desired drug loading (5, 15, and 30% of the intercalated anions in the pristine LDHs were

replaced with RH anions) were prepared by using the anion exchange method [Chai et al., 2008]. For example, to prepare 15% RH intercalated LDH, 1 g LDH was first dispersed in 50 ml of deionized water and was kept in a three-neck round bottom flask followed by degassing through N_2 gas purging for 30 min. 1 M NaOH solution was then added drop wise to the above solutions until the final pH reached to 10. After that, 0.15 g of the drug was added into the above solution and was then stirred vigorously at 55 ^oC for 16 h. The resulting precipitates were centrifuged (6000 rpm), washed thoroughly with ethanol and water, dried at 50 ^oC in air oven and stored. The RH intercalated Mg-Al LDHs were abbreviated as LN-R, LC-R and LP-R using NO_3-LDH, CO_3-LDH and PO_4-LDH, respectively.

2.1.4 Synthesis of Zn-Fe based layered double hydroxides

The Zn-Fe-LDHs with varying interlayer anions (NO_3, CO_3 and PO_4) were synthesized by using coprecipitation method according to previous literature. In brief, 100 mL mixed solution of 0.6 M $Zn(NO_3)_2 \cdot 6H_2O$ and 0.3 M $Fe(NO_3)_3 \cdot 9H_2O$ was added drop wise under nitrogen atmosphere into 100 ml of 1.5 M $NaNO_3$ or Na_2CO_3, or Na_3PO_4 separately and these solutions were stirred vigorously using a magnetic stirrer (rpm 600). 1 M of NaOH solution was added drop-wisely under constant stirring to adjust the pH to 9. The whole solution was stirred continuously at 60 ^oC for overnight. The appearance of brown gelatinous precipitate indicated the formation of Zn-Fe-LDHs (with three different anions intercalated LDHs, NO_3–LDH, CO_3–LDH and PO_4–LDH and were abbreviated as ZN, ZC and ZP, respectively). The precipitates were recovered by centrifugation (6000 rpm, RT) and were washed thoroughly with ethanol and deionized water followed by drying in air oven at 60 ^oC for 36 h.

2.1.5 Intercalation of the drug into Zn-Fe LDHs interlayer gallery

Intercalation of raloxifene hydrochloride into Zn-Fe LDHs interlayer gallery was carried out through anion exchange method. Typically, to prepare 15% RH intercalated Zn-Fe

LDH, 1 g LDH was dispersed in 50 ml of deionized water and was placed in a three-neck round bottom flask followed by degassing the solution through N_2 gas purging and pH was adjusted to 8 by using 1 M NaOH. 0.15 g of the drug was then added into it and was stirred vigorously at 50 ^oC for 16 h. Then, the precipitate was filtered, washed with water thoroughly and dried at 50 ^oC in air oven. The RH intercalated Zn-Fe LDHs were abbreviated as ZN-R, ZC-R and ZP-R using NO_3-LDH (ZN), CO_3-LDH (ZC) and PO_4-LDH (ZP), respectively.

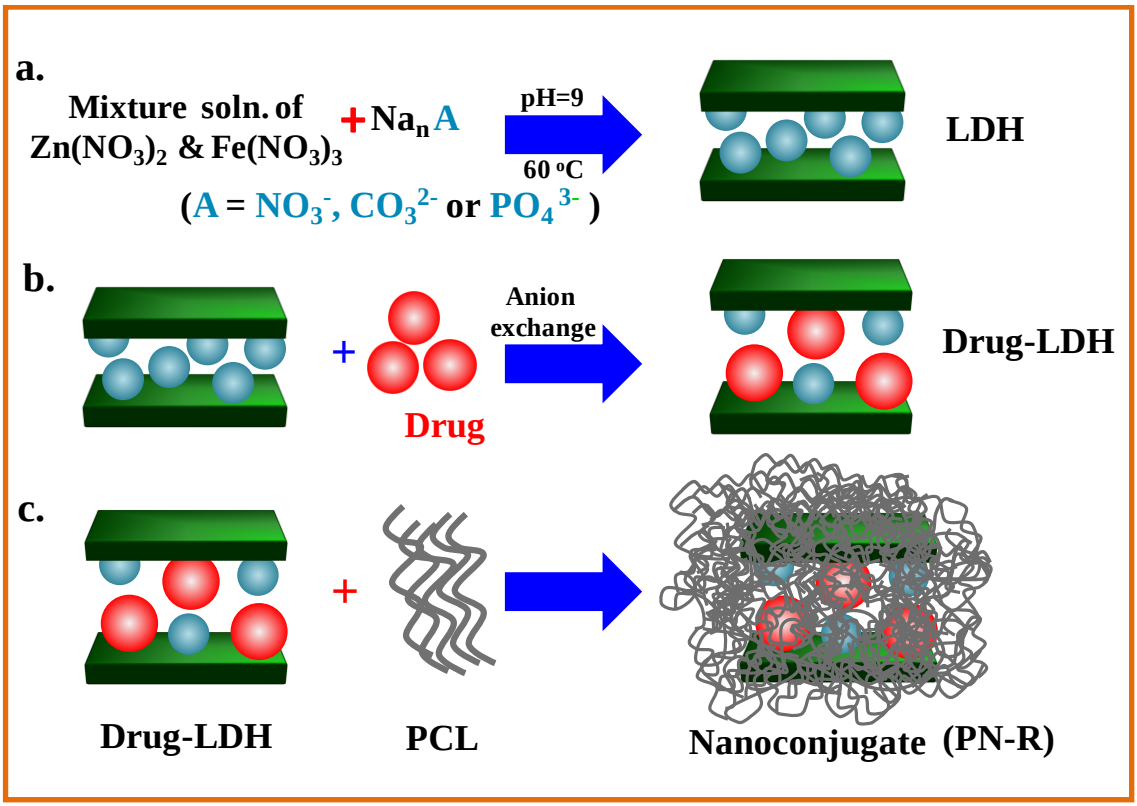

Figure 2.2: (a) Reaction scheme showing synthesis of various ZN-Fe based LDHs with varying interlayer anions; **(b)** intercalation of drug molecules into the interlayer gallery of LDH; and **(c)** dispersion of the drug intercalated LDHs into PCL matrix to form PN-R nanoconjugate.

2.1.6 Preparation of drug embedded polymer nanoconjugate

To prepare drug embedded polymer nanoconjugate, ZN–R was first dispersed in dichloromethane (DCM). PCL was dissolved in DCM separately. ZN–R solution was

then added to the PCL solution (25% ZN–R w/w with respect to PCL) drop wisely and then the whole solution was stirred for 5 h to ensure proper mixing. The mixture solution was then allowed 24 h in a fume hood for solvent evaporation and after that vacuum dried at room temperature for an additional 24 h. We designated this PCL and ZN–R nanoconjugate as PN–R. For comparison, the drug was embedded in pure polymer (PCL) also in the similar way and drug embedded polymer was termed as PCL–RH.

2.1.7 Synthesis of Li-Al layered double hydroxides

The Li-Al nitrate LDH was synthesized by using coprecipitation method. In brief, 75 ml mixed solution of 0.6 M $LiNO_3$ and 0.2 M $Al(NO_3)_3 \cdot 9H_2O$ was added drop wise under nitrogen atmosphere into 50 ml of 1.5 M $NaNO_3$ solution and the mixture solutions was then stirred vigorously using a magnetic stirrer (rpm 600, RT). 1 M of NaOH solution was added drop-wisely under constant stirring to adjust the final pH to 10. The whole solution was stirred continuously at 60 ^{o}C for overnight. The appearance of white gelatinous precipitate indicated the formation of Li-Al-LDH and was abbreviated LA. The precipitate was then recovered by centrifugation (6000 rpm, RT) and washed thoroughlyk with ethanol and deionized water followed by drying in air oven at 60 ^{o}C in air oven for 36 h.

2.1.8 Intercalation of DNA into the interlayer gallery of LDH

The intercalation process was performed by mixing 1 mL salmon sperm DNA stock solution (100 μg ml^{-1}) and certain amount of LDH stock solution (100 μg ml^{-1}) to reach a final w/w ratio of 0.25:1, 0.5:1, 1:1, 1:2, 1:5, and 1:10. The volume of the final mixture solution was fixed as 20 mL by adding water and the final pH was adjusted to 7.4. The mixtures were then vortexed for 1 min followed by stirring at 50 ^{o}C temperature for 24 h. The samples were then centrifuged at 8000 rpm at room temperature followed by drying in air oven at 50 ^{o}C in air oven for 24 h.

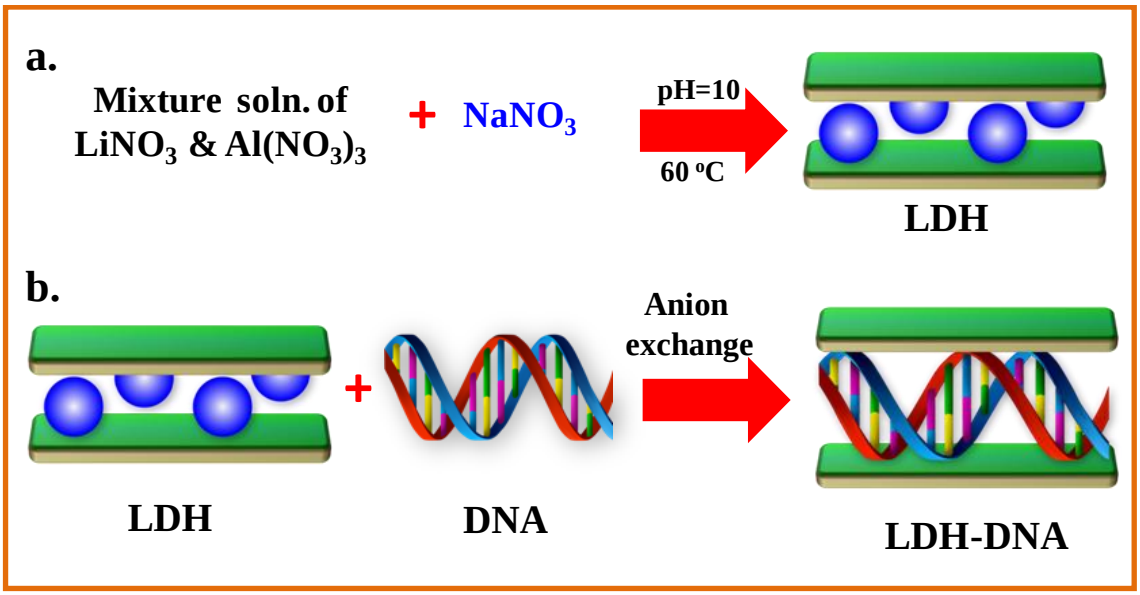

Figure 2.3: (a) Reaction scheme showing synthesis of various Li-Al based LDHs; and **(b)** intercalation of DNA molecules into the interlayer gallery of LDH.

2.1.9 Determination of intercalated drug

Spectrophotometric technique was carried out to determine the amount of intercalated drug into drug-LDH nanoconjugate . A known amount of the drug intercalated LDH was dissolved in 6 M HCl followed by dilution with PBS of pH ~7.4 and the final solution was then stirred at a moderate rate for 6 h at 50 ^{o}C. After that the solution was filtered and the concentration of drug was determined by using UV absorption at 295 nm. The concentration of the drug was determined using a standard curve of known concentrations of the drug.

2.2 Characterization techniques

It is utmost necessary to characterize synthesized various LDHs and their drug/DNA intercalated nanoconjugates and usually are characterized by various spectroscopic and microscopic techniques. Short accounts of these techniques are discussed below:

2.2.1. X-ray Diffraction (XRD)

Powder XRD patterns of the samples were examined to understand the crystal structure of the synthesized LDHs and to confirm the intercalation of drug / DNA molecules into the interlayer galleries of LDHs. Diffraction patterns were recorded by using an advance wide-angle X-ray diffractometer with Cu-Kα radiation and a graphite monochromator (wavelength, $\lambda = 0.154$ nm, Rigaku, MiniFlex-600, Japan). Patterns were recorded at diffraction angle 2θ from 3 to 70^{o} at the scanning rate of 3^{o} min^{-1}.

2.2.2 UV- Vis Spectroscopy

UV-Vis absorption spectroscopy was used to understand the interaction behavior of drug/DNA molecules with LDH host layers and also to calculate the percentage of loaded drug/DNA into LDH interlayer gallery. UV-Visible measurements were performed by using Jasco-V-650 spectrophotometer, Japan, operating in the spectral range of 190 - 1100 nm.

2.2.3 Fourier Transformation Infrared Analysis (FTIR)

Infrared refers to that part of the electromagnetic spectrum between the visible and the microwave regions. FTIR spectroscopy is an important technique based on the interaction of infrared radiation with matter used to identify the presence of functional groups in a sample. FTIR spectra of the samples were obtained in the transmittance mode at room temperature from 400-4000 cm^{-1} on a Thermo Scientific FTIR (NICOLET-6700) with a resolution of 2 cm^{-1} using the KBr pellet technique.

2.2.4 Dynamic light scattering (DLS)

The average particle size (z-average size), its distribution (PDI) and ζ–potential of the pristine LDHs and drug / DNA intercalated LDHs were measured by using Horiba nanoparticle analyzer SZ-100 instrument (Horiba Scientific, Japan) at 25 ^{o}C under a fixed

angle of 90^{o}. All the samples were diluted to a suitable concentration (~0.3 mg/mL) with high purity double distilled water before the measurement.

2.2.5 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy is a surface-sensitive quantitative spectroscopic technique that determines elemental composition, chemical state, empirical formula and amount of interaction between two molecules. XPS measurements of the samples were performed using a VSW made photoelectron spectrometer at UGC-DAE-CSR, Indore, India. The spectra were recorded using a focused monochromatized Al-Kα radiation source with incident energy of 1486.6 eV. Binding energies in all XPS spectra were calibrated using C1s peak (284.6 eV). XPS measurements were conducted under ultrahigh vacuum (~ 4.4×10^{-10} Torr).

2.2.6 Transmission electron microscopy (TEM)

In TEM microscopy the image is obtained from a high energy beam of electrons that are transmitted through a sample. These electrons interact with the sample as they pass through it and thus provide information about the morphology, size and size distribution of the particles.

In this thesis, transmission electron microscope images of pristine LDHs particles and drug / DNA loaded LDH particles were taken using a JEOL JSM-2010 Transmission Electron Microscope (TEM) at the accelerating voltage of 200 kV.

2.2.7 Field emission Scanning Electron Microscopy (FESEM)

The main feature of SEM microscope is that it utilizes high energy electron beams to produce high spatial resolution and high depth of field images of samples because of the secondary electrons. SEM is an important analytical tool which provides important information about the sample's surface topography and elemental composition. The

morphology of the pristine LDHs and its drug intercalated LDHs were investigated by using field emission scanning electron microscopy FESEM ZEISS SUPRA^{TM} 40 instrument operated at 5 kV. All the samples were gold coated by means of a sputtering apparatus under vacuum to make it conducting before observation in FESEM.

2.2.8 Atomic Force Microscopy (AFM)

Atomic force microscopy is a high-resolution type of scanning probe microscopy. AFM provides information about surface morphology, length, thickness and height profile of the sample. In this thesis, AFM analysis was performed in Semi-contact mode at room temperature using Solver scanning probe controlled NT-MDT multimode atomic force microscope, Russia, with the tip mounted on a 100 μm long single beam cantilever with resonant frequency of 240–255 kHz and the corresponding spring constant of 11.5 N m^{-1}.

2.2.9 Contact angle measurement

Contact angle measurement was performed to estimate the hydrophilicity of samples using a Kruss F-100 tensiometer with three specimens of each sample in the form of thin strips (1 × 10 × 15 mm^3) in high purity deionized water.

2.2.10 Thermogravimetric analysis (TGA)

In thermogravimetric analysis, the mass of a substance is monitored as a function of temperature or time as the sample is subjected to a desired temperature program in a controlled atmosphere. The mass loss is used to investigate the decomposition behavior of a sample. The results are generally presented as weight vs. temperature or percentage loss weight vs. temperature. In this thesis work, thermogravimetric analysis (TGA) was studied by using a Mettler-Toledo thermogravimetric/differential thermal analyzer (TGA/DTA). The samples (~ 8 mg) were mounted in an alumina crucible and were heated from 30 to 650 ^{o}C at the heating rate of 20^{o} min^{-1} in nitrogen atmosphere.

2.2.11 Differential Scanning Calorimetry (DSC)

A Mettler DSC was used for studying the melting and crystallization behavior of the samples. Each sample, weighed about 5 mg, was mounted in a platinum crucible and heated at a scan rate of 10^{o} min^{-1} in nitrogen atmosphere. The DSC instrument was calibrated with indium before the study.

2.2.12 Drug assay and release study

Stock solution (1 mg/ml) of the anticancer drug (raloxifene hydrochloride) was prepared first. Standard curve was drawn after taking absorbance measurement using a UV-visible spectrophotometer (Jasco-V-650 spectrophotometer, Japan) taking the absorbance at 295 nm in the concentration range of 1-100 μg/ml. The dissolution test was performed at 37 ^{o}C after suspending drug intercalated nanohybrids in 100 ml phosphate buffer saline (PBS) of pH 7.4. Aliquots (1 ml) of supernatant were taken at regular intervals, and the drug content was determined from the UV-Vis absorption spectra at λ_{max} = 295 nm. Further, to understand the kinetics for the release behavior, various kinetic models have been studied to fit in vitro drug release profiles [Dredán et al., 1996; Costa et al., 2001; Dash et al., 2010]. The mathematical forms of these models are given below:

(1) The zero-order model describes the process of constant drug release from a drug delivery device and can be represented as

$$M_0 - M_t = k_0 t$$

where M_t is the amount of drug dissolved in time t, M_0 is the initial amount of drug in the solution (most times, M_0 = 0) and k_0 is the zero order release constant expressed in units of concentration/time.

(2) The first-order model describes the release from systems where dissolution rate depends on amount of the drug (RH) present in the LDH hybrids and can be generally expressed as

$$\log\ (M_t/M_0\ ) = -k_1 t$$

(3) The parabolic diffusion model express the diffusion controlled release of RH from LDH system and the equation is as follows

$$(1 - M_t/M_0) = k_d t^{-0.5} + b$$

(4) Higuchi model is based on the hypotheses that (i) initial drug concentration in the hybrid is much higher than drug solubility; (ii) drug diffusion occurs only in one dimension; (iii) thickness of the drug particles are much smaller than that of system; (iv) matrix swelling and dissolution are negligible; and (v) drug diffusivity is constant; and can be generally expressed as

$$\mathrm{f_t} = \mathrm{Q} = \mathrm{A}\sqrt{D(2C - C_s)C_s t}$$

where in time t, Q amount of drug is released from per unit area A, C is the drug initial concentration, Cs is the drug solubility in the hybrid media and D is the diffusion coefficient of the drug molecules in the matrix substance.

(5) To investigate the kinetics of drug release, first 60% drug release data were fitted in Korsmeyer-Peppas models and can be represented as

$$\mathrm{M_t/M_\infty} = \mathrm{Kt^n}$$

where Mt / M∞ is a fraction of drug released at time t, k is the release rate constant and n is the release exponent.

(6) The modified Freundlich model explains the release from a flat surface with heterogeneous sites on ion exchange and diffusion-controlled process with the following equation

$$(M_0 - M_t)/M_0 = k_m t^b$$

(7) Elovich model includes a number of processes, bulk and surface diffusion, and the activation/deactivation of catalytic sites and can be expressed using the following equation

$$(1 - M_t/M_0) = k_e \ln(t) + b$$

2.2.13 *In vitro* cell line studies

For any biological applications of newly developed materials, it is necessary that the material should be biocompatible itself while expected to improve the efficacy of the loaded therapeutic agents.

2.2.13.1 Cell Culture and maintenance

HeLa (Human cervical cancer cell line) cells, mouse embryonic fibroblast cell line (NIH 3T3) and murine breast cancer cells 4T1 were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ ml streptomycin. The temperature of the culture was maintained at 37 ^{o}C in a CO_2 incubator with 5% CO_2 supply.

2.2.13.2 Cell viability

The *in vitro* cancer suppression performance of pristine drug and drug intercalated LDHs was investigated against HeLa cell through cell viability test. The percentage of viable cells was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In brief, cells grown to 70-90% confluence onto the 96-well culture plates in 100 µl of serum added DMEM were used for all the samples in triplicate for MTT assay. Pristine drug and drug intercalated LDHs were added to the wells in increasing concentrations from 10 to 100 µg/mL and were incubated for 24, 48, and 72 h time intervals at 37 ^{o}C. After specified time of incubation, media containing drug loaded nanomaterial was replaced with 100 µl fresh medium containing 0.5 mg/ml MTT to each well and was again incubated at 37 ^{o}C for 4 hrs to produce formazan. This purple coloured formazan dye was then dissolved in DMSO and the absorbance was taken using using a microplate reader at 570 nm wavelength. Cellular cytotoxicity of the pristine LDHs was investigated through the MTT assay against NIH 3T3 cell in a similar method mentioned above. The % cell viability was calculated using the formula:

$$\% \text{ cell viability} = \frac{\text{OD of T}}{\text{OD of C}} \times 100$$

where, C is the optical density of 'control' representing HeLa cells incubated in medium alone and T is the optical density of test specimen representing HeLa cells treated with the corresponding samples.

2.2.13.3 Cell adhesion

The cell adhesion behavior of the developed materials was evaluated through a modified crystal violet staining assay protocol and was observed using phase contrast microscope [Patel et al., 2016]. In brief, 1×10^4 cm^{-2} NIH-3T3 and HeLa cells were seeded on the sample surface and incubated for 12 h. The adhered cells were washed twice with PBS, fixed with 4% paraformaldehyde solution for 20 min, washed with PBS again and then cell permeabilization was carried out using 20% methanol for 20 min. The attached cells were then stained by using 0.2% crystal violet aqueous solution. Excess stains were removed by gentle washing in PBS twice followed by elution of the residual crystal violet with 10% acetic acid. Optical density (OD) of the eluted solution was measured using a microplate reader at a wavelength of 570 nm, with the background absorbance value measured at 650 nm. The optical density values thus obtained were correlated directly with the number of attached cells in the sample. Cells adhesion behaviors were also observed using a phase contrast microscope (Leica, Germany) after fixing the cells with 4% paraformaldyhyde solution followed by washing with PBS.

2.2.13.4 Fluorescence imaging

To investigate cancer suppression efficiency of the drug loaded LDHs, 1×10^4 cm^{-2} HeLa cells were seeded in 12 well plate on cover slips as described earlier. Cells were treated with 20 μg/mL solution of drug intercalated LDHs and equivalent amount of pure drug (RH) and were incubated at 37 ^oC in CO_2 incubator for different time intervals. For

pristine materials, the NIH 3T3 cells were seeded at a density of 1×10^4 with various concentrations of LDHs (from 0 to 0.3 mg ml^{-1}). Prior to imaging, the cell culture media was removed and washed three times with PBS. Cells on cover slips were stained with 100 µg/ml Acridine Orange and 100 µg/ml Ethidium Bromide. The images were captured through confocal microscope (LSM780, Carl Zeiss. Germany).

2.2.13.5 Cellular uptake study

The uptake of free drug, pristine LDHs and drug loaded LDHs by NIH-3T3 and HeLa cells was evaluated by both qualitatively and quantitatively. Various LDHs (ZN-Fe and Li-Al based LDHs) surfaces were first modified by grafting the amino silane ((3-aminopropyl)-triethoxysilane, APTES) whose amine groups were then available for the attachment rhodamine-B molecules (RdB). After surface modification with APTES, prepared powder was then dispersed in deionized water, and RdB/EtOH solution was added and finally the mixture was stirred vigorously at room temperature for 16 h. RdB labeled LDH nanoprticles were then collected by centrifugation, washed with water/EtOH, and then freeze-dried.

For the labeling of RH molecules with Rdb, first a known amount of drug (RH) was dispersed in 10 ml of deionized water. A predetermined amount of RdB in water was then added to those dispersion drop wise. The reactions were carried out in dark for 12 h at room temperature with vigorous stirring. After completion of the reaction, RdB labeled RHs were thoroughly washed with ethanol and deionized water. Being a hydrophobic fluorescent dye, the drug molecules were efficiently labeled with rhodamine-B. RdB labeled RHs are abbreviated as RH–RdB. To get PCL nanohybrids of ZN–RdB, first ZN–RdB was dispersed in DCM and followed by its addition to PCL solution in DCM (25% ZN–RdB w/w with respect to PCL) and then the whole solution was stirred for 5 h to ensure proper mixing. The mixture solution was kept for 24 h in a fume hood for solvent

evaporation and then vacuum dried at room temperature for an additional 24 h. This PCL nanohybrids of ZN–RdB is designated as PN–RdB.

The uptake of hybrid nanoparticles by NIH-3T3 and HeLa cells was observed using inverted fluorescence microscopy (DMILLED DFC3000G, Leica, Germany) and quantified by fluorescent intensity measurement through a microplate reader. In brief, 1×10^4 NIH-3T3 and HeLa cells were seeded in 24 well plate for 24 h with 500 μl DMEM consisting of 10% FBS and 100 μg ml^{-1} Penicillin-Streptomycin at 37 ^oC in CO_2 incubator. The solutions containing rhodamine B labeled LDHs and drugs were treated to the cell and incubated for regular time intervals to understand the effect of incubation time to cellular uptake. After desired time of incubation, extracellular materials were removed by washing the cells twice with PBS and then cells were lysed in PBS containing 0.2% Triton X-100, then incubated further for another 15 minutes. The plate was centrifuged to remove cell debris and transfer the supernatant to 96 well plates to measure the fluorescence from rhodamine B at λ_{Ex} = 540 nm and λ_{Em} = 625 nm. For fluorescence imaging cells were incubated for 1, 6, and 24 h and then washed three times with PBS buffer followed by fixation of the cells with 4% paraformaldehyde for 20 min. Fluorescence images of cells with rhodamine B labeled nanohybrids and pure rhodamine B were taken using inverted fluorescence microscopy.

2.2.14 Animal studies

2.2.14.1 Animals

Female Balb/c mice were reared at CSIR-Central Drug Research Institute, Lucknow for the *in vivo* tumor suppression studies and were conducted in accordance with the principles and standard procedures approved by the Institutional Animal Ethics Committee (IEAC) of the CSIR-CDRI, India. Again, 8-10 weeks old female Charles

Foster albino rats, weighing 130±20 g, were purchased for the pharmacokinetics and biochemical parameter analysis from the central animal house of the Institute of Medical Sciences, Banaras Hindu University, India. All the animals were provided a standard laboratory diet and water. The experimental protocol was approved by the animal ethics committee of Banaras Hindu University (Letter No: Dean/2016/CAEC/193) and all the animal studies were carried out in accordance with the approved guidelines and regulations.

2.2.14.2 *In vivo* antitumor efficacy

The *in vivo* cancer suppression potential of the drug loaded LDHs compared to pure drug was evaluated with healthy female Balb/c mice (~ 20 g). Briefly, subconfluent 4T1 cells were trypsinized, washed twice, and harvested by centrifugation at 1000×g for 5 minutes. A single-cell suspension was prepared in phosphate-buffered saline (PBS). Then the cells (1×10^6) were injected subcutaneously into the mammary fat pad of 4-5 week-old female Balb/c mice. When the average tumor volume reached~ 50 mm^3, the mice were divided into six different groups containing of five mice each group: Control (PBS), LN (pristine LDH), RH (free drug), LN–R, LC–R, and LP–R dissolved in PBS. Pristine RH and drug intercalated LDHs were then administered through intra-intraperitoneally (i.p) at 30 mg drug / Kg body weight and equivalent amount in drug intercalated LDH for 21 days having a two days interval between subsequent doses. The tumor volume was calculated using the following formula:

$$\text{tumor volume } (mm^3) = 1/2\ (L \times W \times W),$$

where, L is length and W is width of the tumor. The body weight and tumor volume were recorded every two days after the first injection.

2.2.14.3 Pharmacokinetic study

8-10 weeks old Charles Foster albino female rats, weighing 130±20 g, were procured the central animal house of the Institute of Medical Sciences, Banaras Hindu University, India. They were provided a standard laboratory diet and water. The rats were reared in well ventilated cages at room temperature (22–26 °C) on a regular 12 h dark/light cycle and were maintained relative humidity of 40–60%. Pure drug (RH) and RH intercalated LDHs formulations were injected through intraperitoneally at the dose of 10 mg drug / Kg body weight and equivalent amount in drug intercalated LDHs, and each group consisted of five mice each. Blood samples (~ 0.35 ml) were collected from the supra orbital vein of eye into heparinized microfuge tubes at a time interval of 0.25, 0.50, 1, 2, 4, 8, 16, 24, 30, 48 and 72 h post-dosing. The plasma samples were collected after centrifugation of the collected blood samples at 5,000 rpm for 15 min and stored immediately at –70 ± 10 °C until bioanalysis.

Non-compartmental pharmacokinetics analysis by using Phoenix WinNonlin (version 6.3, Pharsight Corporation, USA) software was employed to determine various pharmacokinetic parameters (such as, area under the curve, AUC; mean elimination half-life, $t1/2$ etc.). Maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were obtained from the observed concentration versus time profiles and area under the plasma concentration versus time curve from time zero to the last quantifiable concentration (AUC_{0-t}) was calculated by using linear trapezoidal rule [Raju et al., 2015].

2.2.14.4 Analysis of biochemical parameters

For biochemical analysis, heparinized blood samples were obtained on day 0 and at the end of treatment by sub mandibular bleeding and stored at about –70 °C until the analysis could be completed. Biochemical analyses included measurement of the activities of

aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), white blood cells (WBC), hemoglobin concentration (Hgb) and platelets (PLT) along with urea and creatinine level using the Hemagen Analyst^{V_R} Benchtop Chemistry System (Hemagen Diagnostics, Inc. Columbia, USA).

2.2.14.5 Histopathological evaluation

Mice and rats were sacrificed and main organs (liver, kidney and spleen) were dissected and fixed in 10% formalin. Sections were immersed in paraffin and cut finely (~ 5-8 μm thickness) using a microtome. The tissues were stained with hematoxylin and eosin (H & E) and monitored using light microscopy at 200× magnification.

2.3 Statistical analysis

Results were presented as the mean value ± standard deviation (SD). Statistical analysis was made using analysis of variance (one-way ANOVA with t-test). P values * < 0.05 were considered significant.