Chapter 2

Experimental

2.1 Synthesis of LDH and Functionalized LDH for sustained drug release

2.1.1 Materials

Lithium nitrate ($LiNO_3$), Aluminium nitrate nonahydrate [$Al(NO_3)_3 \cdot 9H_2O$], and Sodium nitrate ($NaNO_3$) were procured from Merck India Ltd. Poly(tetramethylene glycol) (PTMG), commercially known as Terathane (Sigma-Aldrich), with a number average molecular weight (Mn) of 2900 g/mol, was employed without further purification. 1,6-Hexamethylene diisocyanate (HMDI), along with the catalyst dibutyl tin dilaurate (DBTDL) and the solvent dimethylformamide (DMF), were obtained from Merck, Germany. Essential cell culture reagents, including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), were sourced from Himedia, India. Propidium iodide (PI), Annexin V FITC, and D-luciferin were provided by Thermo Fisher Scientific. Additional reagents, including dimethyl sulfoxide (DMSO), acridine orange, ethidium bromide, and paraformaldehyde, were purchased from Merck India Ltd. Doxorubicin (DOX) was acquired from Neon Laboratories Ltd., India. The human cervical cancer SiHa cell line and the 3T3-L1 fibroblast cell line, derived from mouse embryos, were obtained from the National Centre for Cell Science (NCCS), Pune, India.

2.1.2 Synthesis of Lithium (Li)-Aluminium (Al) based LDHs

Li-Al-based LDHs were synthesized via the coprecipitation method in a custom-designed, five-neck round-bottom flask. The flask was equipped with a gas inlet-outlet, two burettes, a condenser, and a port for a pH meter probe. The synthesis involved varying the molar ratios of Li and Al from lithium nitrate ($LiNO_3$) and aluminium nitrate ($Al(NO_3)_3$), with specific molar percentages of 95:5, 88:12, and 56:44, all carried out under a nitrogen atmosphere. These compositions yielded three distinct nanocarriers, designated as LDH1,

LDH2, and LDH3, respectively. The formation of a white, gelatinous precipitate indicated the successful synthesis of Li-Al-based LDHs. The precipitates were then collected via centrifugation, thoroughly washed with deionized water, and air-dried in an oven at 60°C for 36 hours.

2.1.3 Design of Drug (Doxorubicin) intercalated Li-Al based LDHs nanoformulation

Li-Al based LDHs loaded with the anticancer drug DOX were synthesized using an anion exchange method. DOX (0.268 mg/mL) was dissolved in phosphate-buffered saline (PBS, pH ~7.4) within a three-neck round-bottom flask. The solution was degassed by purging nitrogen gas for 30 minutes, after which sodium hydroxide (1 M) was added to raise the pH of the solution to 9. A suspension of Li-Al based LDHs (0.43 mg/mL) was introduced, and the mixture was stirred continuously for 24 hours. The DOX-loaded Li-Al LDHs were then separated by centrifugation, thoroughly washed with deionized water to remove any unbound Doxorubicin, and subsequently lyophilized to obtain the final drug-intercalated LDHs. These drug-loaded LDHs are referred to as nanoformulations and are labelled as DOX@LDH1, DOX@LDH2, and DOX@LDH3, corresponding to the LDH variants (LDH1, LDH2, and LDH3) used in the synthesis. In the text, the DOX@LDH1, DOX@LDH2, and DOX@LDH3 systems are frequently referred to as nanoformulations or nanovehicles.

2.1.4 Synthesis of polyurethane grafted Lithium (Li)-Aluminium (Al) based LDH nanocomposite

The synthesis of Li-Al based LDH began with a touch of elegance, using the coprecipitation method within a custom-designed, five-necked round-bottom flask. This unique apparatus, masterfully configured, was equipped with a gas inlet-outlet, two burettes, a condenser, and a pH meter probe to meticulously control the reaction environment. The core components

- lithium nitrate ($LiNO_3$) and aluminium nitrate [$Al(NO_3)_3$] - were precisely combined in a molar ratio of 56:44 under a nitrogen atmosphere, leading to the creation of a nanocarrier, denoted as "L". A soft white, gelatinous precipitate gracefully marked the successful formation of the Li-Al based LDH. The precipitate was then carefully collected via centrifugation, thoroughly washed with deionized water, and dried in an air oven at 60°C for 36 hours, yielding the desired nanocarrier. The subsequent synthesis of polyurethane was conducted with equal precision and artistry, unfolding in two distinct stages. The first stage involved the formation of a prepolymer, where the soft segment PTMG was reacted with HMDI for 3 hours at 70°C, resulting in an isocyanate-terminated prepolymer. In the second stage, this prepolymer underwent a chain extension process through the integration of the meticulously prepared Li-Al based LDH nanocarrier. The addition occurred in the presence of a catalyst, DBTDL (0.1 ml of 1 wt% in toluene), and DMF served as the solvent, with vigorous stirring aiding the polymerization. The reaction took place in a well-equipped three-neck flask, fitted with a mechanical stirrer and nitrogen purging inlet, within a silicone oil bath maintained at 70°C for 24 hours.

Upon completion, the polymer mixture was delicately poured into deionized water, a non-solvent, to precipitate the copolymers. Multiple washings ensured the removal of any unreacted dextrin and HMDI, leaving behind a pure product. The polymers were then dried, first in an air oven at 60°C for 24 hours, followed by an additional 24 hours in a vacuum oven, resulting in the final material. This intricate process is visually summarized in the following Scheme, with the grafted polymers being referred to as P-L, where "P" symbolizes the polyurethane prepolymer and "L" represents the integrated nanocarrier.

Scheme 1 (Page -150): The synthesis of a Polyurethane-grafted LDH (P-L) nanocomposite unfolds through a fascinating reaction mechanism, where polyurethane (P) chains elegantly graft onto a LDH (L) framework. This stepwise process involves the gradual attachment of the polyurethane chains to the backbone of the LDH, leading to the formation of a robust and thermally stable nanocomposite.

NOTE: Initially, the hydroxyl groups present on the surface of the LDH interact with reactive isocyanate groups from the polyurethane precursor. Through a nucleophilic addition, the isocyanate groups react with the hydroxyls, forming urethane linkages that tether the polyurethane chains onto the layered structure. As the reaction progresses, multiple polyurethane chains become anchored, yielding a grafted architecture with enhanced mechanical properties. The result is a novel P-L nanocomposite, where the interfacial bonding between the organic polyurethane matrix and the inorganic LDH imparts remarkable thermal stability and mechanical strength. This innovative material holds significant promise for advanced applications, merging the flexibility of polyurethane with the structural integrity of LDH into a seamless hybrid composite.

2.1.5 Synthesis of the doxorubicin drug intercalated polyurethane grafted Li-Al based LDH nanocomposite system

A drug-loaded grafted nanocomposite was meticulously synthesized through an anion exchange method, incorporating doxorubicin (Dox) into the nanocomposite matrix. The process began with dissolving 5 wt% of Dox, relative to the nanocomposite, in dimethylformamide (DMF) within a three-neck round-bottom flask. This solution was carefully degassed by purging with nitrogen gas for 30 minutes. To achieve a pH of approximately 8, a 1 M sodium hydroxide solution was then added to the drug solution. In a separate step, the grafted nanocomposite was dissolved in DMF in a known proportion,

before being introduced into the flask containing the drug solution. The mixture was gently stirred at room temperature for 24 hours to ensure the thorough integration of the drug with the nanocomposite, facilitating its complete encapsulation within the matrix. Upon completion of the blending process, the solution was lyophilized, producing a Dox-intercalated nanocomposite film, referred to as the P-L@Dox system. This formulation is frequently referenced as such throughout the main text.

2.2 Materials Characterization

2.2.1 X-ray photoelectron spectroscopy (XPS)

XPS measurements were performed using a VSW photoelectron spectrometer, utilizing Al-Kα radiation with an incident energy of 1350 eV. The binding energies across all XPS spectra were calibrated against the C1s peak at 284.6 eV. These measurements were conducted under ultrahigh vacuum conditions, with a pressure of approximately 4.4×10^{-10} Torr, ensuring precise and reliable data acquisition.

2.2.2 Particle size and zeta potential (Dynamic Light Scattering, DLS)

The average particle size (z-average size) and zeta potential of the nanocarriers were measured using a Horiba nanoparticle analyzer SZ-100 (Horiba Scientific, Japan). The analysis was conducted at a controlled temperature of 25°C, with a fixed angle of 90°, utilizing high-purity double-distilled water as the dispersing medium. The concentration of the samples was maintained at approximately 0.3 mg/ml to ensure accuracy in the results.

2.2.3 X-ray diffraction (XRD)

The Powder XRD analysis was carried out using a Rigaku MiniFlex-600 instrument (Japan), equipped with Cu-Kα radiation and a graphite monochromator ($\lambda = 0.154$ nm).

The diffraction patterns were recorded over a 2θ range from 3° to 70°, at a scanning rate of 3° per minute.

2.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis was conducted in transmittance mode for both the Li-Al based LDHs nanocarrier and the DOX-loaded Li-Al based LDHs nanoformulation. The spectra were acquired at room temperature, spanning the range from 400 to 4000 cm^{-1}, using a Thermo Scientific FTIR (NICOLET-6700) with a resolution of 2 cm^{-1}. The samples were prepared using the KBr pellet method to ensure optimal spectral quality.

2.2.5 UV- Vis Spectroscopy

The UV-Visible measurements for the Li-Al-based LDHs nanocarrier, along with the doxorubicin (Dox) intercalated Li-Al-based LDHs nano formulation, were conducted using a Jasco V-650 Spectrophotometer from Japan. This sophisticated instrument operates across a spectral range of 190 to 1100 nm, providing precise insights into the optical properties of the nanomaterials.

2.2.6 Nuclear magnetic resonance (NMR)

NMR spectroscopy for both ^{1}H and ^{13}C was conducted using the AVH D 500 AVANCE III HD 500 MHz OneBay NMR Spectrometer (BRUKER BioSpin INTERNATIONAL AG, Germany). The analysis was performed over a spectral width of 0–20 ppm, comprising 512 scans, utilizing DMF-d6 (99.98%) as the solvent.

2.2.7 Thermal study

DSC: The melting and glass transition temperatures of the samples were ascertained through meticulous thermal analysis. Initially, the samples were swiftly quenched from room temperature to −50°C at a controlled cooling rate of 30°C per minute. Following this

rapid cooling, they were gradually heated to 200°C at a rate of 10°C per minute using a Mettler 832 differential scanning calorimeter (DSC). The heat of fusion (ΔH) during the melting process was derived from the area beneath the distinctive endothermic peak. To ensure precision, the DSC was carefully calibrated with indium before measurements were taken, guaranteeing the utmost accuracy in the results.

TGA: The thermal analysis of the samples was conducted using a Mettler-Toledo Thermogravimetric/Differential Thermal Analyzer (TGA/DTA). Approximately 7 mg of both prepolymer and grafted polyurethane were carefully placed in an alumina crucible. The heating process spanned from 25°C to 600°C, with a rate of 20°C per minute, all under a controlled nitrogen atmosphere.

2.2.8 Mechanical Study

The mechanical properties of the prepolymer, reference molecule, and grafted polyurethane were meticulously examined using an INSTRON 3369 tester. Samples, measuring (15×8×2) mm3 were fabricated, and the experiments were conducted at a constant strain rate of 5 mm/min. To ensure the reliability of the results, each test was repeated three times, minimizing the potential for error and enhancing the accuracy of the data collected. Key parameters analyzed included tensile stress, elongation at break, elastic modulus, and toughness.

2.2.9 Morphological investigations

To capture exquisite images of LDH nanomaterials, a JEOL JSM-2010 TEM was employed, operating at an accelerating voltage of 200 kV. The morphology of pure LDHs, their composites, and drug-intercalated systems was meticulously examined using AFM and high-resolution field emission scanning electron microscopy (HRSEM). The latter was conducted with a ZEISS SUPRATM 40 instrument, operating at 5 kV, allowing for a

detailed analysis of the thin film's surface morphology. Before FESEM observation, all samples underwent sputter-coating with gold to enhance imaging quality. For the AFM analysis, a single beam cantilever measuring 100 μm in length, with a resonance frequency ranging from 240 to 255 kHz, was carefully mounted on the NT-MDT multimode atomic force microscope. This instrument operated in tapping mode, utilizing a spring constant of 11.5 N m^{-1} ensuring precise topographical measurements.

2.2.10 Brunauer-Emmett-Teller (BET)

BET measurements were conducted using the Belsorp Max II instrument from Japan at IIT (BHU) in Varanasi. This sophisticated apparatus meticulously analyzed the surface area and pore size distribution of the designed nanocarriers, providing invaluable insights into their structural characteristics.

2.2.11 Rheological analysis

The rheological parameters, including Storage Modulus, Loss Modulus, and complex viscosity, of P and P-L were meticulously analyzed using an Anton Paar MCR 702 (multidrive) Rheometer. This analysis employed a parallel-plate geometry with a diameter of 25 mm, conducted at a controlled temperature of 160°C. Throughout the measurement process, a constant gap of 1 mm was diligently maintained between the plates, ensuring precise and reliable results.

2.2.12 Dynamic Mechanical Analysis (DMA)

The dynamic mechanical analysis of P and P-L was conducted utilizing an Anton Paar MCR 702 (multidrive) DMA. For this purpose, samples measuring 25 × 10 × 2 mm^3 were

meticulously prepared. The experiments were carried out across a temperature range of -100 to 100 °C, maintaining a constant frequency of 1 Hz and a strain amplitude of 15 μm. The heating rate was set at 3 °C/min, ensuring precise and consistent results throughout the analysis.

2.2.13 Computational study

In this study, we employ first-principles calculations rooted in DFT to delve into the electronic structure and bonding interactions between surface-modified lithium aluminium hydroxide ($LiAl(OH)_4$) and the anticancer drug doxorubicin. Our objective is to assess the potential of these surface-modified LiAl-based LDHs as innovative drug delivery systems for cancer therapy. Initially, we conducted calculations utilizing various force fields (FF). However, during the process of variable cell optimization, we observed a loss of co-planarity among the cations, attributed to the lack of suitable LDH-specific force fields (LDHFF) for Li^+ ions. This limitation may significantly impact our analysis of cation ordering. Similar structural alterations in the octahedral sheets have been documented in previous theoretical studies employing molecular dynamics with force fields. To address these challenges, we turned to quantum mechanical calculations based on DFT. The calculations were performed under three-dimensional periodic boundary conditions and plane-wave settings using the Quantum Espresso software package. We modelled the exchange-correlation potential utilizing Bayesian estimation-based BEEF-vdW parameters. Structural analyses of the crystal system were executed with VESTA software, while ionic pseudo-potentials were treated using the projector-augmented-wave (PAW) method. A Monkhorst-Pack grid of 4 × 4 × 1 was employed to sample the reciprocal space, with a plane wave energy cutoff set to 400 eV for all supercells, ensuring that the total energy of the system converged within 1 meV. The criteria for energy cut-off and convergence were established at 1×10^{-5} eV and 0.001 eV/Å, respectively.

For mechanical calculations of the bulk systems, we utilized CHGNET, a graph neural network-based potential. The deformations applied to the bulk crystals in this study were defined by a series of strains generated using the Class Deformed Structure Set from the pymatgen package. By default, this method imposes several small deformations on the original structure, which are typically employed to compute the independent elastic constants of the crystal. Each strain applied is characterized by the Green-Lagrange strain tensors, with the magnitude of deformation varying according to the default strain range of the Deformed Structure Set, generally around 1-2% for each independent strain. Such modest deformations are typically adequate for extracting accurate elastic properties, such as the elastic tensor, without inducing non-linear effects that might significantly alter the material's mechanical behaviour.

2.3 *In vitro* drug release study

The comparative study of doxorubicin (DOX) release from the nanoformulation was meticulously evaluated through UV absorption measurements, utilizing an excitation wavelength of 480 nm. At predetermined intervals, 3 mL samples of the incubation solutions were carefully extracted and replaced with an equal volume of fresh buffer medium to maintain constant conditions. The drug release was assessed at three distinct pH levels: 7.4, 6.0, and 4.0. To gain deeper insights into the release dynamics, detailed kinetic models were applied, allowing for a comprehensive understanding of the release behaviour, which was subsequently fitted to various in vitro drug release profiles.

2.4 Detailed *in vitro* study

2.4.1 Cell culture and viability study

SiHa cells, derived from human cervical cancer, and 3T3-L1 cells, a fibroblast line isolated from mouse embryos, were cultivated in Dulbecco's Modified Eagle Medium (DMEM).

This medium was enriched with 10% heat-inactivated fetal bovine serum, along with 100 µg/ml of penicillin and 100 µg/ml of streptomycin. The cultures were maintained at 37°C in a CO_2 incubator, providing a stable environment with 5% CO_2. To investigate the *in vitro* anti-tumor effects of LDHs, nanocomposite, pristine drugs, and drug-intercalated different systems against both SiHa and 3T3-L1 cells, a cell viability assay was performed. The MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was employed to determine the percentage of viable cells. Each treatment was assessed in triplicate on 96-well culture plates with a cell density of 1×10^5 cells in 0.1 ml per well. The nanoformulations, along with pristine DOX and LiAl-based LDH nanocarriers, were introduced into the wells at a concentration of 20 µg/ml. After a predetermined incubation period, the media containing the treatments were replaced with fresh medium supplemented with 0.5 mg/ml MTT for each well. This was followed by an additional 4-hour incubation at 37°C, leading to the formation of purple formazan crystals dissolved in DMSO. The absorbance of the resulting solution was then measured using an Amersham spectrophotometer at a wavelength of 570 nm. The percentage of cell viability was calculated using the formula:

$$\% \text{ Cell viability} = (\text{Optical density of Test})/(\text{Optical density of control}) \times 100$$

2.4.2 Cell adhesion

The cell adhesion behavior of pristine nanocarriers, alongside nanocomposite and drug-intercalated carriers, was meticulously evaluated through a modified crystal violet staining assay using a phase contrast microscope. A density of 1×10^4 SiHa / 3T3-L1 cells per cm^2 was inoculated onto the sample surfaces and cultured for 24 hours. Following this incubation period, unattached cells were gently removed from the specimens by performing two washes with phosphate-buffered saline (PBS). The adhered cells were subsequently

fixed for 15 minutes in a 4% paraformaldehyde solution to preserve their structural integrity. To facilitate further analysis, the cells were permeabilized with 20% methanol for 20 minutes after a thorough PBS wash. The adhered cells were then stained with a 0.2% crystal violet aqueous solution (Himedia, India) for 20 minutes, allowing for clear visualization of cell adhesion. Any excess stain was carefully washed away with two gentle PBS rinses, and the remaining crystal violet was eluted using 10% acetic acid. The optical density (OD) of the eluted solution was measured at a wavelength of 570 nm using a microplate analyzer (BioTek, USA), with the resulting values correlating directly to the number of adhered cells. Furthermore, the adhesion behavior of the cells was observed using a phase contrast microscope (Leica, Germany) after fixation with the paraformaldehyde solution and subsequent PBS washing, providing a comprehensive understanding of the interaction between cells and the various carrier systems.

$$\% \text{ Cell adhesion} = (\text{Optical density of Test})/(\text{Optical density of control}) \times 100$$

2.4.3 Fluorescence imaging

SiHa cells were cultured in a 12-well plate on cover slips, reaching a confluence of 1×10^4. The cells were incubated at 37 °C in a CO_2 incubator, treated with nanocarriers/drug-loaded nanoformulations at a concentration of 20 µg/ml for designated time intervals of 24, 48, and 72 hours. Following treatment, the adherent cells were fixed in 4% paraformaldehyde for 15 minutes. Subsequently, they were stained with a fluorescent dye mixture of acridine orange and ethidium bromide (0.1 mg/ml) for 10 minutes. Excess stain was washed away using phosphate-buffered saline (PBS), and images were captured with a fluorescent microscope (Leica, Germany).

2.4.4 Cellular uptake

Inverted fluorescence microscopy (DMILLED DFC3000G, Leica, Germany) was employed to investigate the cellular uptake of pristine doxorubicin (DOX) and its nanoformulations in SiHa cells. Fluorescent intensity was quantified using a microplate reader to assess drug absorption. For the experiment, a 24-well plate was prepared by seeding 2×10^4 SiHa cells and incubating them for 24 hours in 500 µl of DMEM supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml of penicillin and streptomycin. To explore the effects of incubation duration on cellular absorption, both pristine DOX and drug-loaded nanoformulations were introduced to the cells, followed by incubation for 1, 3, 6, 12, 24, and 48 hours. After each incubation period, extracellular components were removed by rinsing the cells with phosphate-buffered saline (PBS). The cells were subsequently lysed in PBS containing 0.2% Triton X-100 and incubated for an additional 20 minutes at 37 °C. The lysates were then centrifuged to eliminate cell debris, and the supernatant was transferred to 96-well plates for fluorescence measurement, with excitation at λ_{Ex} = 480 nm and emission at λ_{Em} = 590 nm. Cells were treated for 1, 3, 6 and 12 hours, rinsed three times in PBS, and then fixed for 15 minutes at room temperature with 4% paraformaldehyde. Finally, inverted fluorescence microscopy was utilized to capture fluorescent images of the cells, revealing the presence of both pristine DOX and its nanoformulations.

2.4.5 Intracellular tracking

SiHa cells were cultured in 6-well plates at a density of 1×10^5 cells per well to investigate the intracellular tracking of a novel formulation. The cells were maintained in serum-free DMEM media containing formulations at a concentration of 20 µg/mL for varying durations. Following this, the cells were incubated for 45 minutes in serum-free DMEM

media supplemented with 100 nM LysoTracker Green DND-26, after which they were washed with PBS. To preserve cellular morphology, the cells were fixed using 4% paraformaldehyde for 20 minutes, rinsed with PBS, and subsequently stained with 5 µg/mL DAPI for an additional 20 minutes. The stained cells were then examined under a fluorescent microscope (Leica, Germany) following thorough PBS washes.

2.4.6 Cell cycle analysis using Flow cytometry

In this study, we meticulously evaluated various stages of the cell cycle following therapy with drug-intercalated nanovehicles, employing the precision of flow cytometry. SiHa cells were initially seeded overnight and subsequently treated with pristine DOX, along with a range of novel nanoformulations at a concentration of 20 µg/ml for a duration of 24 hours. Post-treatment, the cells were thoroughly washed, fixed in chilled 70% ethanol, and stored at 4 °C for 30 minutes. To prepare for analysis, the cells were stained for 30 minutes in the dark. This process involved the addition of 50 µl of a 100 µg/ml RNase stock solution, ensuring that only DNA was stained, followed by the incorporation of 200 µl of propidium iodide (PI) from a 50 µg/ml stock solution. The stained cells were then analyzed using a Beckman Coulter instrument, with channel B585, while CytExpert software facilitated comprehensive data processing. We determined the percentages of viable, early apoptotic, late apoptotic, and necrotic cells after the administration of drug-intercalated Li-Al based LDH nanovehicles. After overnight attachment, the SiHa cells were exposed to a 20 µg/ml dose of DOX and nanoformulations for 24 hours. For further characterization, the cells underwent staining with Annexin V/PI, adhering to the manufacturer's instructions following a PBS wash. Subsequent analysis was conducted with the Beckman Coulter instrument, utilizing channel B-525 FITC for Annexin V and Y585 PE for PI, capturing ten thousand events to ensure robust data. The resulting data was processed using CytExpert

software, revealing critical insights into the cellular response to the therapeutic interventions.

2.4.7 Analysis of protein expression through Western blotting

The impact of doxorubicin (DOX) and various novel nanoformulations on the expression of apoptotic protein markers was meticulously investigated using Western blotting techniques. SiHa cell lines were cultured in high-glucose DMEM, supplemented with 10% fetal bovine serum (FBS), and treated with penicillin (100 units/ml) and streptomycin (100 μg/ml) to ensure optimal growth conditions. These cells were maintained in a controlled environment with 5% CO2 and were subjected to treatment with different samples for 24 hours, with some cases extending to 72 hours. Following the treatment period, the cells were harvested and washed with phosphate-buffered saline (PBS) to prepare whole cell lysates. Protein quantification was performed, and equal amounts of protein (25-30 μg/ml) were loaded onto SDS-polyacrylamide gels for electrophoresis. The separated proteins were then transferred onto nitrocellulose membranes for further analysis. To probe for specific proteins, the membranes were incubated with a selection of primary antibodies, including monoclonal anti-pro-Caspase 3 (1:1000), polyclonal anti-Bcl-XL (1:1000), polyclonal anti-p53 (1:1000), and polyclonal anti-PARP (1:1000). Additionally, monoclonal anti-β-actin (1:1000) was utilized as a loading control, followed by HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies for detection. Ultimately, enhanced chemiluminescence (ECL) reagent was employed to visualize the protein signals, allowing for a comprehensive analysis of apoptotic markers.

2.5 Animal Studies

2.5.1 *In vivo* anti-tumor efficacy

The Institute of Medical Sciences (IMS) at Banaras Hindu University (BHU), India, provided six to seven-week-old mice for an innovative study on subcutaneous tumors. These tumors were induced by injecting 1×10^6 B16-F10 cells with luciferase and without luciferase into the right flank of the mice. In this experiment, groups of three mice each received subcutaneous injections at the tumor site, with one group receiving saline as a control, while another was treated with pristine doxorubicin (DOX) in MC gel at a dosage of 5 mg/kg. Additionally, a third group was administered 5 mg/kg of DOX intercalated in Li-Al based LDH nanocarrier and composite systems encapsulated in MC gel. These injections were administered every three days over a span of 27 days, beginning once the tumor volume reached approximately 50 ± 10 mm^3 /25 ± 5 mm^3 , utilizing an injectable hydrogel formulation. Every third day, the tumor volume was meticulously measured with a vernier calliper, using the formula: tumor volume = (length × $width^2$)/2. Throughout the study, the tumor volume and body weight of the mice in all three groups were recorded, allowing for a comprehensive analysis over time. The study received ethical approval from the Institutional Animal Ethics Committee (IAEC) at the Indian Institute of Technology, Banaras Hindu University, Varanasi, under IAEC Approval Number: IIT(BHU)/IAEC/2023/050, ensuring strict adherence to ethical regulations for the use of animals in research (Regd. No. 2123/GO/Re/S/21/CPCSEA).

2.5.2 *In vivo* biodistribution along with pharmacokinetics study

Blood samples were collected following the intravenous administration of three groups: DOX and various novel formulation groups, each receiving an equivalent dose of 5 mg/kg. Samples were taken at predetermined time points of 0.5, 1, 3, 6, 12, 24, 36, and 48 hours

post-injection. The collected blood samples were then centrifuged at 10,000 rpm for 10 minutes at 4 °C, allowing the supernatant to be isolated and dried. To prepare for analysis, 50 µL of a mobile phase - comprising a freshly prepared mixture of acetonitrile and water in a 1:1 volume ratio—was added to the dried samples. In parallel, B16 F10 tumor-bearing mice were injected at the tumor site with pristine DOX and DOX@LDH3 hydrogel, also at an equivalent dose of 5 mg/kg, to assess the distribution of the drug across various organs. After 24 hours post-injection, the mice were sacrificed, and vital organs - including the heart, liver, spleen, lungs, and kidneys - along with the tumors, were harvested. These tissues were homogenized to extract DOX by incubating them in methanol containing 5% HCl for 12 hours. Following this, the mixture was centrifuged at 12,000 rpm for 10 minutes, and the supernatant containing DOX was collected, dried at room temperature, and subsequently reconstituted with 50 µL of the mobile phase. In both cases, 20 µL of each sample was injected into a C18G reverse phase column at a flow rate of 1.3 mL/min. Analysis was conducted using a PDA detector set to a wavelength of 480 nm, with a retention time of approximately 2.1 minutes at 35 °C. This methodology allowed for the evaluation of dose kinetics and the quantification of drug concentrations in various organs and tumor tissues.

2.5.3 Histopathology along with biochemical assessment study

At the conclusion of the experiment, mice were humanely sacrificed, and their major organs, along with tumor tissues, were meticulously collected and immersed in a 10% formalin solution for various studies. To prepare the samples for analysis, dehydration was performed using a series of graded ethanol solutions, followed by embedding the tissues in paraffin wax. The specimens were then sliced into sections of 3-5 µm thickness and subjected to hematoxylin and eosin (H&E) staining. These stained sections were

subsequently examined under a light microscope at a specified magnification, revealing intricate details of the tissue architecture.

Additionally, blood samples, with a volume of 50 µl per mouse, were collected for the clinical assessment of several critical blood parameters. These included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine, ensuring a comprehensive evaluation of the physiological impacts observed throughout the study.

2.5.4 Immunohistochemistry

To begin the preparation, fresh xylene was employed for a meticulous five-minute deparaffinization of the slides. This was followed by a careful hydration process, where the slides were immersed in sequential solutions of ethanol: 100%, 95%, 80%, 70%, and 50%, each for five minutes, ensuring thorough penetration. The slides were then washed twice with distilled water, each wash lasting five minutes, followed by a final cleansing in distilled water for another five minutes. Next, the slides were treated with tris buffer solution (TBS) for five minutes to effectively quench any endogenous peroxidase activity. This step was complemented by a ten-minute exposure to a 3% hydrogen peroxide solution. After this, the slides were placed in a heated jar for five minutes to facilitate further processing. Washing was completed using a phosphate buffer solution. With the region of interest marked, protein blocking was applied at room temperature for twenty minutes. Once the blocking solution was removed, 25 µl of the primary antibodies—TNF-α (bs-0078R, Bioss Antibodies Company) and CD31 (MA5-37858, Thermo Fisher Scientific Company)—were introduced, each diluted to 1:100 for individual experiments. The slides were then carefully stored overnight in a petri dish at 4 °C to allow the antibodies to bind effectively. Following this incubation, the slides were rinsed with TBS and treated with

secondary antibodies, incubating at 37 °C for twenty minutes before being rinsed with phosphate-buffered saline (PBS). Subsequently, a DAB solution was applied for one minute, and the slides were washed with distilled water. A brief counterstaining with hematoxylin followed for another minute. Once dried, the slides were mounted with DPX. Finally, the beautifully stained sections were captured using a Leica microscope, a premier optical instrument from Germany, allowing for a detailed examination of the results.

2.6 Statistical Analysis

The standard deviation (SD) was utilized to represent the data as the mean value. To evaluate differences among the groups, a one-way ANOVA was performed, complemented by Tukey's tests. A significance level of $*p < 0.05$ was considered statistically significant, while $**p < 0.01$ and $***p < 0.001$ were deemed extremely significant.