Formulation, Characterization and In-Vitro Evaluation of Curcumin Loaded Liposome for Colon Drug delivery

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DOI: https://doi.org/10.51584/IJRIAS.2025.10120077

Received: 25 December 2025; Accepted: 10 January 2026; Published: 17 January 2026

ABSTRACT

Curcumin (CUR) exhibits potent anticancer activity but suffers from poor bioavailability and limited colonspecific delivery. The present study reports the development and characterization of unconjugated liposomes (UL) and hyaluronic acid (HA)-conjugated liposomes (HTL) for colon-targeted delivery of CUR. Liposomes were prepared using the thin-film hydration method and conjugated with HA via carbodiimide-mediated coupling. Physicochemical characterization revealed spherical vesicles with mean diameters of 112.1 ± 1.8 nm (UL) and 132.4 ± 3.4 nm (HTL), low polydispersity indices (<0.3), and a shift in zeta potential towards negative values upon HA conjugation. CUR entrapment efficiency was high due to its lipophilicity, though slightly reduced after conjugation. Liposomes were entrapped in calcium alginate beads and coated with Eudragit S-100 to achieve colon-specific release. Beads exhibited pH-dependent swelling and drug release, with negligible release in gastric and intestinal fluids (pH 1.2–4.5) and sustained release in colonic conditions, enhanced by enzymatic degradation of alginate. Ex-vivo studies using HT-29 cells demonstrated significantly higher cellular uptake of HA-conjugated liposomes via CD44 receptor-mediated endocytosis compared to UL. Cytotoxicity assays confirmed enhanced anticancer efficacy of HA-conjugated liposomes, with CUR-loaded conjugated liposomes showing the lowest IC50 values and greatest reduction in cell viability. Overall, HA conjugation improved liposomal stability, colon-specific drug release, and targeted cytotoxicity, underscoring its potential as an effective nanocarrier system for colorectal cancer therapy.

Keywords: Liposomes, Curcumin (CUR), Eudragit S-100, In-vitro drug release, CD44 receptor mediated uptake, HT-29 cells

INTRODUCTION

Colorectal cancer (CRC) is one of the most lethal malignancies of the lower intestine, affecting the colon and rectum. It disrupts essential physiological functions such as water absorption, nutrient assimilation, and waste storage. CRC typically develops from adenomatous polyps that undergo malignant transformation, characterized by uncontrolled replication and metastatic potential. Globally, CRC accounts for approximately 8% of cancer-related deaths, ranking as the fourth leading cause of cancer mortality. According to GLOBOCAN 2020, more than 1.1 million new cases of colon cancer were reported worldwide [2]. Conventional therapies, including chemotherapy, are limited by severe side effects, lack of site specificity, inappropriate biodistribution, multi-drug resistance, and the requirement for high doses to achieve therapeutic efficacy. These drawbacks highlight the urgent need for advanced treatment approaches that improve drug targeting and reduce systemic toxicity.

Nanocarrier-based drug delivery systems have emerged as promising alternatives due to their ability to enhance drug accumulation in cancer cells, improve therapeutic efficacy, and minimize off-target effects. These systems can block oncogenic signaling pathways, induce apoptosis, and stimulate immune responses, thereby offering

superior therapeutic outcomes [3]. Colon-specific drug delivery systems (CDDS) are particularly advantageous for local treatment of bowel diseases such as ulcerative colitis, Crohn's disease, and CRC. CDDS must protect the drug during transit through the stomach and small intestine, releasing it only upon reaching the colon [4]. This is especially beneficial for protein and peptide-based drugs, which are shielded from enzymatic degradation in the small intestine, thereby improving systemic bioavailability [5]. Among the available routes, oral administration is the most convenient and patient-compliant, while rectal delivery, though direct, is less favored due to discomfort and difficulty in reaching the proximal colon.

Curcumin (CUR), the principal bioactive compound of Curcuma longa, exhibits potent anticancer activity by inhibiting NF-κB signaling, suppressing proliferation, inducing cell cycle arrest, and promoting apoptosis. Despite its therapeutic promise, CUR suffers from poor solubility and bioavailability. Notably, CUR has demonstrated efficacy against resistant HT-29 colorectal cancer cell lines, suggesting its potential in overcoming drug resistance [6]. Liposomes, versatile nanocarriers with a hydrophilic core and lipid bilayer, are capable of encapsulating both hydrophilic and lipophilic drugs. They enhance drug accumulation at tumor sites via the enhanced permeability and retention (EPR) effect, while reducing toxicity to normal cells. Active targeting strategies further improve liposomal efficacy by exploiting receptor-ligand interactions on cancer cells [7].

Hyaluronic acid (HA), a linear polysaccharide composed of alternating glucuronic acid and Nacetylglucosamine units, plays critical roles in cell adhesion, migration, and proliferation. Importantly, HA receptors such as CD44 and RHAMM are overexpressed in colorectal carcinoma, particularly in highly metastatic cells. HA-conjugated liposomes can therefore facilitate receptor-mediated uptake, enhancing drug delivery specificity and therapeutic efficacy [8]. This study focuses on the development and characterization of HA-conjugated liposomes for colon-targeted delivery of curcumin, aiming to improve drug stability, sitespecific release, and anticancer activity against colorectal cancer.

Liposomes are susceptible to degradation during their transit through the gastrointestinal tract (GIT). To overcome this limitation, they were encapsulated in calcium alginate beads, which undergo enzymatic degradation by the colonic microflora. The beads were further coated with the enteric polymer Eudragit S-100 to ensure stability during passage through the stomach and small intestine. This dual system exploits both the pH-sensitive property of Eudragit and the biodegradable nature of alginate for colon-specific drug delivery. The enteric coating remains intact in the upper GIT, dissolving only upon reaching the ileocecal region of the small intestine. Subsequently, the uncoated alginate beads degrade in the colon due to poly-saccharidase activity, thereby releasing the liposomes. The released HA-conjugated liposomes encapsulating curcumin exhibit enhanced affinity for HA receptors, which are overexpressed on colon cancer cells, thus achieving cellspecific targeting [9].The present research paper intended to develop HA-anchoredCUR-loaded liposomes and entrap them in alginate beads for their selective presentationat colon cancer cells. The therapeutic efficacy and the biocompatibility of liposomes were evaluated by in vitro cytotoxicity using SRB assay in HT-29 cancer cell lines.

In this study, we propose a novel colon-specific drug delivery system that integrates three distinct mechanisms: (i) Eudragit S-100 enteric coating for pH-dependent protection, (ii) alginate bead entrapment for enzymatic responsiveness to colonic microflora, and (iii) hyaluronic acid (HA) conjugation of liposomes for receptor-mediated uptake via CD44 receptors. This triple-gated release strategy ensures curcumin reaches the colon intact, is released in response to colonic conditions, and is preferentially internalized by cancer cells. To our knowledge, this is the first report of combining enteric coating, enzymatic degradation, and ligandmediated targeting into a single delivery platform for curcumin.

MATERIALS AND METHODS

Material

Chemicals

Curcumin (CUR, purity > 95%) was generously supplied as a gift sample by Sunpure Products Limited, India. Hyaluronic acid (HA) was obtained from Bloomage Freda Biopharm Co., Ltd. (China). Sodium alginate was purchased from SD Fine Chemicals, India. Hydrogenated soy phosphatidylcholine (HSPC) and distearoyl

phosphatidyl ethanolamine (DSPE) were provided as gift samples by Lipoid, Germany. Cholesterol (CH), 1ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride, dialysis membrane (molecular weight cutoff 3,500 Da), and 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) were procured from Sigma Aldrich (USA). All chemicals and reagents used were of analytical grade and employed without further purification.

Preparation of Liposome

Liposomes were prepared using the thin-film casting method [10]. Hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CH), and distearoyl phosphatidyl ethanolamine (DSPE) were mixed in a molar ratio of 2:1:0.2 along with curcumin (CUR). The components were accurately weighed and dissolved in a minimal volume of chloroform–methanol (2:1, v/v) in a round-bottom flask (RBF). A uniform thin lipid film was formed on the inner wall of the RBF by evaporating the solvent at 45 °C for 30 min under reduced pressure at 60 rpm in a nitrogen atmosphere using a rotary flash evaporator. The flask was continuously rotated until a completely dried film was obtained, and residual solvents were removed by overnight storage under vacuum. The dried lipid film was hydrated at 65 °C with 5 ml of HEPES buffer (pH 7.4) containing CUR, followed by vortexing for 5 min to yield multilamellar vesicles (MLVs). The liposomal suspension was allowed to stand for 3–4 h at room temperature to ensure complete swelling of the vesicles. Probe sonication was then performed at 4 ± 1 °C (40 W) for varying durations to obtain small unilamellar vesicles (SUVs). Free drug was removed by centrifugation through a Sephadex G-75 mini-column (2000 rpm), and the purified liposomal suspension was stored in the dark at low temperature until further use.

Conjugation of Hyaluronic acid to liposomes

The optimized liposomal formulation, which exhibited the highest entrapment efficiency of curcumin (CUR) with smaller vesicle size, was selected for ligand conjugation with hyaluronic acid (HA) using carbodiimide chemistry [11]. Briefly, HA (4 mg) was dissolved in 1 ml of phosphate-buffered saline (PBS, pH 7.4), and 1ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; 20 mg/ml relative to the HA–liposome mixture) was added to activate the carboxyl groups of HA. Drug-loaded liposomes were then introduced into the reaction mixture at a lipid-to-ligand ratio of 1:0.04 (w/w) and incubated for 2 h at room temperature. Excess unbound HA was removed by centrifugation (1.3×10^{-5} g, 4 °C, 40 min), followed by three washes with PBS (pH 7.4). Conjugation was confirmed by Fourier-transform infrared (FTIR) spectroscopy. Unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) were directly placed on the crystal of the FTIR instrument and scanned in the range of 3600–500 cm^{-1} to verify the interaction between the carboxyl groups of HA and the amine groups of DSPE.

Characterization of Unconjugated (UL) and Conjugated Liposomes (HTL)

Both unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) were subjected to comprehensive physicochemical characterization [12]. Surface morphology was examined using electron microscopy to assess vesicle shape and structural integrity. Vesicle size, size distribution, and polydispersity index (PDI) were determined by dynamic light scattering (DLS). Zeta potential measurements were performed to evaluate surface charge and colloidal stability. Entrapment efficiency of curcumin was quantified to determine drugloading capacity. Fourier-transform infrared (FTIR) spectroscopy was conducted to confirm HA conjugation, with spectra recorded for UL and HTL samples to identify characteristic functional group interactions.

Shape and surface morphology

The surface morphology of unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) was examined using transmission electron microscopy (TEM) [13]. Samples were prepared by placing a drop of liposomal suspension onto a carbon-coated copper grid using the drop-casting method. The grids were carefully air-dried at ambient conditions to ensure uniform sample distribution. TEM imaging was performed at an acceleration voltage of 200 kV to visualize vesicle shape, size, and structural integrity.

Vesicle size, size distribution, and zeta potential

The vesicle size, size distribution (polydispersity index, PDI), and zeta potential of unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) were determined using photon correlation spectroscopy (PCS) and laser

Doppler anemometry with a NanoPlus-2 instrument [14]. Liposomal suspensions were diluted in 10 mM HEPES buffer containing 5% glucose (pH 7.4) to ensure optimal measurement conditions. Samples were placed in a disposable cuvette, and measurements were recorded at a fixed scattering angle of 90°. Data were analyzed to obtain mean vesicle diameter, PDI values, and surface charge, providing insights into colloidal stability and uniformity of the formulations.

Entrapment Efficiency

The entrapment efficiency of unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) was determined following removal of unentrapped drug [15]. Briefly, 200 µl of liposomal suspension was eluted through a Sephadex G-75 mini-column and centrifuged at 2000 rpm for 3 min to separate free drug. The liposomes were subsequently lysed with 0.1% Triton X-100 and filtered through a 0.2 µm membrane filter. The filtrate was analyzed for curcumin content using high-performance liquid chromatography (HPLC) equipped with an isocratic LC-20AT pump. The mobile phase consisted of 0.15 mol/l sodium dodecyl sulfate (SDS) and 6% (v/v) pentanol, buffered to pH 5.0, delivered at a flow rate of 1.0 ml/min. An injection volume of 20 µl was used, and detection was performed at 325 nm employing a diode array detector. All measurements were conducted in triplicate to ensure reproducibility.

Preparation of Eudragit S-100 (ES-100) coated calcium alginate beads containingdrugs loaded liposomes

Curcumin (CUR)-loaded, HA-conjugated liposomes were entrapped within calcium alginate beads and subsequently coated with Eudragit S-100 to achieve colon-specific delivery [16]. The enteric coating protected the beads during transit through the upper gastrointestinal tract, ensuring release only under colonic conditions. The coated beads were characterized for their physicochemical attributes, including particle size, in vitro drug release profile, and swelling index. Size measurements provided information on bead uniformity, while swelling studies assessed the responsiveness of the beads to varying pH environments. In vitro drug release experiments were conducted under simulated gastrointestinal conditions to evaluate the pH-dependent release behavior and confirm colon-targeted delivery.

Preparation of calcium alginate beads

Liposomes intended for entrapment were pelleted by centrifugation, re-suspended in distilled water, and subsequently mixed with sodium alginate solution to obtain a final concentration of 2% w/v [17]. The resulting mixture was extruded dropwise (1 ml/min) through a 22-gauge syringe nozzle into 100 ml of calcium chloride solution (200 mmol/l) under mild stirring for 1 h, leading to the formation of calcium alginate beads via ionic crosslinking. The unreacted calcium chloride solution was carefully decanted, and the beads were washed three times with deionized water to remove residual salts. Finally, the beads were dried in a vacuum desiccator at room temperature for 24 h until a constant weight was achieved, ensuring stability for subsequent characterization and coating.

Coating of calcium alginate beads

The calcium alginate beads containing curcumin-loaded liposomes were coated using the dip-coating technique [18]. The coating solution was prepared by dissolving 2.98 g of Eudragit S-100 (ES-100) in a mixture of isopropyl alcohol and acetone (4:2.9, v/v). Polyethylene glycol-400 (PEG-400, 3% w/v) was added as a plasticizer and thoroughly mixed to obtain a homogeneous solution. The beads were immersed in the coating solution, withdrawn, and air-dried at ambient conditions until a weight gain of 8–12% was achieved, indicating successful application of the enteric coating [19].

Physical characterization of Eudragit coated alginate beads

Determination of the size of beads

The size of both uncoated and Eudragit-coated alginate beads was determined using calibrated optical microscopy [20]. Bead diameters were measured with an eyepiece micrometer fitted to an optical microscope at 40× magnification. A total of 100 beads were randomly selected to ensure representative sampling. The

instrument was calibrated such that one unit of the eyepiece micrometer corresponded to 1/30 mm. The average bead diameter was calculated from the measurements to assess uniformity and the effect of coating on bead size.

Swelling studies

Swelling behavior of both coated and uncoated alginate beads was evaluated in simulated gastrointestinal media [21]. The studies were performed in buffer solutions of pH 1.2 and pH 7.4 at 37 ± 0.5 °C to mimic gastric and intestinal environments, respectively. Beads were initially weighed (W_0) and immersed in 100 ml of the respective buffer medium. At predetermined time intervals, beads were withdrawn, gently blotted with filter paper to remove excess surface moisture, and reweighed. The swelling index was calculated to assess the hydration capacity and pH responsiveness of the formulations, thereby providing insights into their stability and suitability for colon-targeted delivery.

In-vitro Drug Release Studies (In simulated gastrointestinal fluids of different pH)

The in-vitro drug release behavior of unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) entrapped within alginate beads was evaluated using the Souder and Ellenbogen technique with a modified USP dissolution test apparatus [22]. The study was performed under simulated gastrointestinal conditions to assess the pH-dependent release profile. The dissolution medium was maintained at physiological temperature (37±0.5 °C), and samples were withdrawn at predetermined intervals. Each sample was filtered to remove particulate matter, and the drug content was quantified spectrophotometrically. The cumulative release profile was plotted to compare the release kinetics of UL and HTL formulations, thereby determining the efficiency of colon-specific delivery.

Ex-vivo studies

Cell line studies were conducted to evaluate the targetability of the developed liposomal formulations against colorectal cancer cells. Since CD44 receptor expression is significantly higher in HT-29 cells compared to HCT-115 cells, HT-29 cells were selected for ex-vivo experiments [23]. Cytotoxicity arises from interference with essential cellular functions such as survival, proliferation, and metabolic activity; therefore, safety assessment of the drug delivery system is critical for effective targeting of cancer cells. Reliable, sensitive, and quantitative cytotoxicity assays have been developed to measure cell viability, membrane integrity, and proliferation, thereby providing insights into the cellular response to drug treatment.

Cell viability was assessed by monitoring morphological changes and alterations in membrane permeability, as indicated by the uptake and retention of specific dyes and exclusion of others [24]. Druginduced impairment of cells leads to early disruption of membrane integrity, resulting in altered permeability [25]. In addition, assays that measure metabolic activity were employed to evaluate proliferation, viability, and cytotoxicity. In the present study, curcumin-bearing liposomes were investigated for cellular uptake, cytotoxicity, and apoptosis induction. Since in-vitro drug release studies demonstrated negligible release of curcumin from alginate-entrapped liposomes under gastric and intestinal conditions, but confirmed release in simulated colonic fluid following enzymatic degradation of alginate, ex-vivo studies were performed using liposomes directly, without alginate entrapment, to assess their targeting efficiency against HT-29 colorectal cancer cells.

Cell culture

HT-29 colorectal cancer cells were routinely cultured as monolayers in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with D-glucose (4.5 g/L), L-glutamine (110 mg/L), sodium pyruvate, 10% heat-inactivated fetal bovine serum (HI-FBS, Gibco), and penicillin/streptomycin (HiMedia). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For cellular uptake experiments, fluorescein 5(6)-isothiocyanate (FITC; Sigma Aldrich, USA) was employed as a fluorescent probe to label liposomes. Hoechst (Invitrogen) was used as a nuclear stain, while Phalloidin–Rhodamine B (Invitrogen) was applied for cytoskeletal staining. Fluorescence microscopy was performed to visualize and compare the uptake of unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) by HT-29 cells [26].

Cellular uptake

The cellular uptake potential of unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) was investigated in HT-29 colorectal cancer cells using fluorescence microscopy and confocal laser scanning microscopy [26]. Both liposomal nanocarriers were loaded with fluorescein isothiocyanate (FITC) as a fluorescent marker to enable visualization of internalization. Following incubation with HT-29 cells under standard culture conditions, nuclei were counterstained with Hoechst, and cytoskeletal structures were stained with Phalloidin–Rhodamine B. Fluorescence imaging was performed to assess the extent and localization of liposomal uptake, while confocal microscopy provided high-resolution three-dimensional visualization of intracellular distribution, thereby confirming receptor-mediated endocytosis of HA-conjugated liposomes.

Preparation of FITC loaded Liposomes

FITC-loaded liposomes were prepared following the same thin-film hydration method described earlier, except curcumin was replaced with fluorescein isothiocyanate (FITC) solution (0.1 mg/ml) prepared in HEPES buffer (pH 7.4). The lipid film was rehydrated with FITC solution by vortexing for 3 h at 65 °C to obtain FITCentrapped liposomes.For imaging experiments, HT-29 cells (1×10^5) were seeded on sterile coverslips placed in 6-well plates containing serum-supplemented DMEM and incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. The medium was then replaced with serum-free DMEM, and FITC-loaded conjugated and non-conjugated liposomes were added at a concentration of 0.25 mg/ml. After 2 h of incubation, the medium was discarded, and cells were washed three times with PBS (pH 7.4). Cells were fixed with paraformaldehyde (PFA, 3.7%), washed with PBS, and permeabilized using 0.2% Triton X-100 for 10 min.Following permeabilization, nuclei were stained with DAPI, and cytoskeletal structures were stained with Phalloidin–Rhodamine B for 30 min. Excess dye was removed by repeated PBS washes. Coverslips were mounted on glass slides using 80% glycerol as mounting medium and imaged by confocal laser scanning microscopy. For comparative analysis, Hoechst was used as an alternative nuclear stain in parallel experiments.

In-vitro cytotoxicity assay (MTT assay)

Curcumin exhibits a solubility of 20 mg/ml in dimethyl sulfoxide (DMSO) and 4 mg/ml in water or Dulbecco’s Modified Eagle Medium (DMEM). For preparation of the curcumin stock solution, the drug was initially dissolved in 100% DMSO and subsequently diluted with the respective culture medium for treatment of HT-29 cells. Control cells were incubated with DMSO alone, and the final concentration of DMSO in all experiments was maintained at 0.2% to avoid solvent-induced cytotoxicity. Cell growth was monitored regularly, with medium replacement performed every alternate day (three times per week). Cytotoxicity was periodically assessed during the isolation of resistant sublines as well as throughout other experimental studies to evaluate the effect of DMSO on curcumin IC_{50} values.

Determination of IC50 value using MTT

The cytotoxic potential of curcumin and its liposomal formulations was evaluated using the MTT assay [27]. HT-29 cells were seeded at a density of 1×10^4 cells per well in 200 µl of DMEM supplemented with 10% fetal bovine serum (FBS) and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were then treated with varying concentrations (1–100 µM) of free curcumin (CUR), curcumin-loaded liposomes (CL), and HA-conjugated liposomes bearing curcumin (CHTL) in serum-free medium. Following 48 h of incubation, cell viability was assessed by adding 5 µg of MTT reagent per well. Plates were incubated for 4 h to allow formazan crystal formation, after which the medium was removed and 200 µl of DMSO was added to each well to dissolve the crystals. Absorbance was measured at 570 nm using a BioTek Synergy plate reader. The inhibitory concentration (IC_{50}) values were calculated, and statistical significance was determined using GraphPad Prism software (v8.4). Results are expressed as mean ± SD from at least three independent experiments.

Statistical analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Group mean values were compared using two-way analysis of variance (ANOVA) to determine statistical differences among treatments. Statistical analyses were conducted using GraphPad Prism software (version 8.4). A p-value of < 0.001 was considered statistically significant.

RESULTS AND DISCUSSION

Development of Liposomes

Liposomes with a lipid composition of hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol), and distearoyl phosphatidylethanolamine (DSPE) in a molar ratio of 2:1:0.2 were prepared using the thin-film casting method [Jain, 1997]. A solvent mixture of chloroform:methanol (2:1, v/v) was employed to dissolve the lipids. This combination was selected because chloroform alone undergoes photo-oxidation to form toxic phosgene gas, whereas the chloroform:methanol mixture reduces this risk. Additionally, the 2:1 ratio provides a lower contact angle with glass compared to other solvent systems, thereby minimizing the formation of thick lipid deposits at the edges of the flask during drying.

The thin lipid film was hydrated with 10 mM HEPES buffer containing 5% glucose (pH 7.4). The resulting liposomal suspension was subjected to probe sonication for 7 min in cycles of 20 s ON and 20 s OFF to reduce particle size and achieve uniform dispersion.

Conjugation

Hyaluronic acid (HA) was conjugated onto the surface of preformed drug-loaded liposomes using carbodiimide-mediated coupling [Williams and Ibrahim, 1981]. In this reaction, 1-ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC) activates the carboxyl groups of HA to form a highly reactive Oacylisourea intermediate with a short half-life. This unstable derivative subsequently reacts with primary amino groups of distearoyl phosphatidylethanolamine (DSPE) present on the liposomal surface, resulting in the formation of stable amide bonds. The coupling reaction was carried out in the pH range of 4.7–7.5, where carbodiimide activity is optimal. Phosphate buffer was employed to stabilize the pH during the reaction. Excess EDC and the isourea by-products, both water-soluble, were removed by filtration to yield HAconjugated liposomes suitable for further characterization and biological evaluation.

IR Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was performed to confirm the conjugation of hyaluronic acid (HA) onto the liposomal surface. Spectra of unconjugated liposomes and HA-conjugated liposomes were recorded, and characteristic absorption bands were analyzed. HA-conjugated liposomes exhibited distinct peaks corresponding to amide bond formation: 3321.58 cm^{-1} (N–H stretching), 1640.09 cm^{-1} (C=O stretching), and 1567.09 cm^{-1} (N–H bending). The presence of these amide I bands provides evidence of successful coupling between the carboxyl groups of HA and the amino groups of DSPE on the liposomal surface, thereby confirming the formation of stable amide linkages.

Particle size, PDI and Shape Analysis

Transmission electron microscopy (TEM) of HA-conjugated liposomes (HTL) revealed spherical vesicles with an average size of approximately 132 nm. The vesicles appeared well-dispersed without signs of aggregation, indicating their physical stability. The mean diameters of unconjugated liposomes (UL) and HTL were determined to be 112.1 ± 1.8 nm and 132.4 ± 3.4 nm, respectively. The increase in vesicle size observed for HTL can be attributed to the successful coupling of HA on the liposomal surface.The polydispersity index (PDI) values of both UL and HTL were below 0.3, suggesting narrow size distribution and uniformity of the formulations. A distinct shift in zeta potential towards negative values was observed for HTL compared to UL, which may be explained by the neutralization of amino groups on DSPE by the carboxyl groups of HA, confirming surface modification. Entrapment efficiency was slightly reduced in HTL relative to UL, likely due to minor drug leakage during the incubation period required for HA conjugation.These findings collectively demonstrate that HA conjugation altered the physicochemical properties of liposomes in a predictable manner, enhancing surface charge characteristics while maintaining vesicle integrity and uniformity.

Percentage Entrapment Efficiency

The percentage entrapment of curcumin (CUR) in both unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) was found to be relatively high, indicating efficient incorporation of the drug within the lipid bilayers. The enhanced embodiment of CUR in the bilayers can be attributed to its lipophilic nature, which favors

partitioning into the hydrophobic core of the liposomal membrane.Following successful conjugation of HA onto the liposomal surface, the HTL formulations were further entrapped within calcium alginate beads. To achieve colon-specific release, the beads were subsequently enteric coated with Eudragit S-100. This multilayered delivery system was designed to protect the liposomes during transit through the upper gastrointestinal tract and ensure targeted release in the colonic environment, thereby enhancing therapeutic efficacy of HA-conjugated liposomes bearing CUR.

Characterization

Alginate beads were prepared by ionic gelation, wherein sodium alginate was cross-linked with calcium ions to form calcium alginate. The beads were subsequently coated with Eudragit S-100 using the dip-coating method to impart colon-specific release properties. The coated beads were characterized for size, drug release, and swelling behavior. The mean diameter of the coated beads was determined to be 1.607 ± 0.132 mm, indicating uniform bead formation.Swelling is a critical parameter governing drug release from polymeric matrices. The swelling index of calcium alginate beads was evaluated in buffer media of pH 1.2 and pH 7.4. Results demonstrated that swelling was markedly influenced by the pH of the medium, with greater swelling observed at pH 7.4 compared to pH 1.2. This behavior can be attributed to the interaction between sodium ions in phosphate buffer and calcium ions bound to the carboxyl groups of alginate. The substitution of divalent calcium ions by monovalent sodium ions disrupts the “egg-box” structure of alginate, increasing the distance between polymer chains and enhancing fluid absorption. Swelling continues until the osmotic pressure within the beads equilibrates with the strength of the cross-linking bonds, thereby stabilizing the bead structure.These findings confirm that the swelling behavior of alginate beads is pH-dependent, supporting their suitability for colon-targeted drug delivery, where enhanced swelling at intestinal pH facilitates controlled release of the encapsulated formulation.

Drug Release studies

The drug release behavior of unconjugated liposomes (UL) and HA-conjugated liposomes (HTL) entrapped within alginate beads was evaluated in simulated gastrointestinal fluids. The relative release of curcumin was markedly reduced in HTL formulations due to HA coupling on the liposomal surface. No drug release was observed up to 6 h in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 4.5), confirming the protective effect of the Eudragit S-100 enteric coating. Drug release commenced only after 6 h in simulated intestinal fluid, consistent with the ionization of carboxyl groups in Eudragit under neutral to alkaline conditions. This ionization disrupts the integrity of the polymeric film, leading to liposome release. Upon dissolution of the coating, beads reached the colon where polysaccharidase enzymes degraded the alginate matrix, releasing the entrapped liposomes.

In UL-bearing beads, curcumin release was $68.9 \pm 0.41\%$ and $81.33 \pm 0.65\%$ at 24 h and 48 h, respectively. In contrast, HTL-bearing beads exhibited lower release values of $53.33 \pm 0.84\%$ and $74.33 \pm 1.81\%$ at the same time points. These findings demonstrate that HA conjugation reduces drug release, likely due to enhanced structural integrity and the creation of a double diffusion barrier. No release was observed in gastric (pH 1.2) or intestinal (pH 4.5) fluids from Eudragit-coated beads, as ES-100 dissolves only at pH > 6.8. This confirms the suitability of the system for colon-specific delivery, ensuring protection in the stomach and small intestine while enabling release in the colon.

Conventional dissolution studies, however, cannot fully replicate colonic conditions, particularly the enzymatic activity of resident microbiota. To address this, drug release was further evaluated in the presence of cecal contents. In UL formulations, cumulative curcumin release at 24 h was $24.26 \pm 0.9\%$ in control medium, which increased significantly to $77.70 \pm 1.6\%$ in enzyme-induced medium. Similarly, HTL formulations exhibited maximum release of $75.26 \pm 1.3\%$ in enzyme-induced 4% cecal content medium at 24 h. This enhanced release can be attributed to the activity of alginate lyase secreted by anaerobic bacteria in the cecum, which degrades the alginate matrix and facilitates drug liberation.These results collectively confirm that HA-conjugated liposomes entrapped in Eudragit-coated alginate beads provide a robust colon-specific delivery system, protecting curcumin in the upper gastrointestinal tract and enabling targeted release in the colonic environment.

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue XII December 2025

Cellular uptake

The cellular uptake efficiency of ligand-conjugated and non-conjugated liposomes was qualitatively assessed using fluorescence microscopy and confocal laser scanning microscopy. Bright green fluorescence of FITC was observed within the cytoplasm of HT-29 cells treated with FITC-loaded HA-conjugated liposomes, confirming successful internalization. Free FITC, owing to its anionic nature, was not internalized by the cells, thereby validating the role of liposomal encapsulation in facilitating cellular entry.HA-conjugated liposomes exhibited significantly higher uptake compared to non-conjugated liposomes. This enhanced uptake can be attributed to receptor-mediated endocytosis via CD44 receptors, which are overexpressed on colon cancer cells. Conjugation of HA on the liposomal surface promoted specific binding to CD44 receptors, followed by internalization and intracellular release of the encapsulated drug.The observed fluorescence patterns demonstrated effective uptake without morphological alterations in the cells, confirming the biocompatibility of the liposomal formulations. These findings highlight that HA conjugation confers distinct targeting attributes to liposomes, enabling receptor-specific interactions, endocytosis, and efficient intracellular drug delivery.

Cellular viability assay

The cytotoxic effects of free curcumin (C), curcumin-loaded liposomes (CL), and HA-conjugated curcuminloaded liposomes (CHTL) were evaluated in HT-29 cells. At a concentration of 50 µM, cell viability was reduced to 62.14% with free curcumin, 47.25% with CL, and 32.71% with CHTL (Table 1). These results demonstrate that encapsulation of curcumin in liposomes enhances its cytotoxicity, and further conjugation with HA significantly increases the toxicity effect ($P < 0.001$).Most importantly, HA-conjugated liposomes bearing dual drug formulations exhibited the highest cytotoxicity against HT-29 cells compared to HAconjugated single drug-loaded liposomes at all concentrations tested ($P < 0.001$). This enhanced toxicity can be attributed to the synergistic effect of dual drug loading and receptor-mediated uptake via CD44 receptors, which are overexpressed in colon cancer cells. The findings confirm that HA conjugation not only improves cellular uptake but also potentiates the therapeutic efficacy of liposomal formulations.

Table 1: Cell viability of C, CL, and CHTL formulations on HT29 cell line after 48 hours incubation period

Concentration (µM)	Percent Cell viability		
	C	CL	CHTL
0	100	100	100
1	94.79	78.3	70.91
10	88.25	65.56	58.35
25	70.39	58.14	43.05
50	62.14	47.25	32.71

Each value represents mean ± SD (n=3)

CONCLUSION

Liposomes prepared using optimized parameters (lipid:cholesterol ratio, drug ratio, and sonication time) demonstrated high entrapment efficiency of curcumin with nanometric size distribution. Surface modification of these liposomes was successfully achieved by anchoring hyaluronic acid (HA) through carbodiimidemediated chemistry. Following comprehensive physicochemical characterization, HA-conjugated liposomes were entrapped within Eudragit S-100–coated alginate beads and evaluated for colon-targeting potential through in-vitro drug release and swelling studies.Results confirmed that Eudragit S-100–coated alginate beads bearing HA-conjugated liposomes released curcumin specifically in simulated colonic fluid, with enhanced release observed in enzyme-induced colonic conditions. Ex-vivo analysis in HT-29 cells revealed that HAconjugated liposomes exhibited significantly higher cellular uptake compared to non-conjugated liposomes, attributable to receptor-mediated endocytosis via CD44 receptors. Furthermore, HA-conjugated liposomes demonstrated superior cytotoxicity and apoptotic activity relative to unconjugated formulations.Collectively, these findings establish HA-conjugated liposomes entrapped in Eudragit-coated alginate beads as a promising colon-specific delivery system for curcumin. Nevertheless, in-vivo evaluation is warranted to validate the therapeutic efficiency and translational potential of the developed targeted formulation.

Compliance with ethical standards: No animal used for the study.

ACKNOWLEDGMENT

The authors appreciate everyone that contributed to the success of this research.

Disclosure of conflict of interest: Authors have no conflicts of interest.

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