



Original Research Paper

CELLULAR UPTAKE AND INTRACELLULAR TRAFFICKING OF SOLID LIPID NANOPARTICLES FOR ENHANCED DELIVERY OF GLECAPREVIR/PIBRENTASVIR

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ABSTRACT

Background: Glecaprevir and pibrentasvir are potent direct-acting antivirals (DAAs) approved for hepatitis C virus (HCV) therapy; however, their clinical utility is constrained by poor aqueous solubility and suboptimal bioavailability. Solid lipid nanoparticles (SLNs) have emerged as promising nanocarriers for enhancing the delivery of lipophilic drugs. While our previous work established the physicochemical characteristics and *in vitro* release profile of glecaprevir/pibrentasvir-loaded SLNs, the cellular mechanisms governing their uptake and intracellular trafficking remain unexplored.

Objective: This study investigates the cellular uptake mechanisms, intracellular trafficking pathways, and endosomal escape behavior of glecaprevir and pibrentasvir co-loaded SLNs in hepatocyte models to elucidate the enhanced delivery potential of this nanocarrier system.

Methods: Fluorescently labeled SLNs were prepared using the microemulsion technique with rhodamine 123 as a probe. Cellular uptake studies were performed in HepG2 cells using flow cytometry and confocal laser scanning microscopy. Pharmacological inhibitors were employed to delineate endocytic pathways: chlorpromazine (clathrin-mediated), filipin (caveolae/lipid raft-mediated), amiloride (macropinocytosis), and sodium azide (energy-dependent). Intracellular trafficking was tracked using organelle-specific fluorescent markers for early endosomes (Rab5), late endosomes (Rab7), lysosomes (Lamp-1), and endoplasmic reticulum. Endosomal escape was assessed using the pH-sensitive dye LysoTracker and colocalization analysis.

Results: SLN uptake was time- and concentration-dependent, reaching plateau at 4 hours. Mechanistic studies revealed that internalization occurred primarily via clathrin-mediated endocytosis (52.3% inhibition with chlorpromazine, $p < 0.01$), with minor contributions from caveolae/lipid raft-mediated pathways (31.7% inhibition with filipin) and macropinocytosis (24.8% inhibition with amiloride). Uptake was significantly reduced at 4°C and in the presence of sodium azide, confirming energy dependence. Intracellular trafficking analysis demonstrated that SLNs sequentially localized to early endosomes (15 min), late endosomes (60 min), and lysosomes (120 min), with a significant fraction escaping endosomal compartments and localizing to the cytoplasm by 240 min. Colocalization with LysoTracker showed $68.4 \pm 5.4\%$ escape efficiency at 4 hours. The encapsulated drugs exhibited enhanced intracellular accumulation compared to free drug solution (3.2-fold increase, $p < 0.001$).

Conclusion: Glecaprevir/pibrentasvir-loaded SLNs exploit clathrin-mediated endocytosis as the primary uptake mechanism, followed by efficient endosomal escape and sustained intracellular drug release. These findings establish a mechanistic basis for the enhanced bioavailability and therapeutic potential of SLN-based delivery of DAAs in HCV therapy.

Keywords: Solid lipid nanoparticles, Glecaprevir, Pibrentasvir, Cellular uptake, Endocytosis, Intracellular trafficking, Endosomal escape, HCV therapy

1. INTRODUCTION

Hepatitis C virus (HCV) infection remains a major global health burden, affecting approximately 58 million people worldwide and serving as a leading cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma [1]. The

introduction of direct-acting antivirals (DAAs) has revolutionized HCV management, with the fixed-dose combination of glecaprevir and pibrentasvir (Mavyret™) emerging as a highly potent, pangenotypic regimen approved by the USFDA [2]. Glecaprevir, a NS3/4A protease inhibitor, and pibrentasvir, a NS5A inhibitor, act

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synergistically to block viral replication and have demonstrated sustained virologic response rates exceeding 95% across all major HCV genotypes [3].

Despite their remarkable therapeutic efficacy, the clinical performance of glecaprevir and pibrentasvir is significantly hampered by their physicochemical limitations. Both drugs are highly lipophilic with poor aqueous solubility, resulting in suboptimal oral bioavailability [4]. Additionally, extensive first-pass metabolism, high protein binding, and potential drug-drug interactions via CYP3A4 and P-glycoprotein further complicate their pharmacokinetic profile [5]. These challenges necessitate advanced formulation strategies to achieve therapeutic plasma concentrations and improve patient outcomes [6].

The biocompatibility of advanced biomaterial-based delivery systems is an important consideration in their development and therapeutic application. *In vitro* evaluation provides valuable information regarding the biological compatibility of such biomaterials before their further application in drug delivery systems [7].

Solid lipid nanoparticles (SLNs) have emerged as one of the most promising nanocarrier systems for lipophilic drugs. Composed of biocompatible solid lipids stabilized by surfactants, SLNs offer several advantages, including enhanced drug solubility, improved bioavailability through lymphatic uptake, controlled and sustained drug release, protection of encapsulated drugs from degradation, and excellent physical stability [8,9]. SLNs have been successfully explored for the delivery of various antiviral agents, demonstrating improved therapeutic efficacy and reduced dosing frequency [10,11].

Our previous study successfully developed and characterized glecaprevir and pibrentasvir co-loaded SLNs using the microemulsion technique [6]. The optimized formulation (F3) exhibited a mean particle size of 148.6 ± 5.2 nm, PDI of 0.21 ± 0.03 , zeta potential of -28.4 ± 1.8 mV, and entrapment efficiencies of 92.4% for glecaprevir and 89.7% for pibrentasvir. *In vitro* release studies demonstrated sustained biphasic release over 48 hours, and the formulation remained stable under ICH conditions for 90 days. While these findings established the physicochemical superiority of the SLN formulation, the cellular mechanisms underlying its enhanced delivery potential remained unexplored.

Understanding the cellular uptake mechanisms and intracellular trafficking of nanocarriers is crucial for rational design and optimization of drug delivery systems [12,13]. The mechanisms by which nanoparticles enter target cells can differ depending on their size, surface properties, and composition [14]. SLNs are known to breach the cell-membrane barrier through various endocytic pathways, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and lipid raft-mediated routes [15, 16].

The endocytosis of SLNs has been reported to be time-dependent and energy-dependent. However, few studies have systematically investigated the uptake mechanisms and intracellular trafficking of SLNs in hepatocytes, particularly for antiviral drug delivery [17].

Once internalized, nanoparticles typically traffic through the endolysosomal pathway, where they may be subjected to acidic pH and enzymatic degradation. Endosomal escape is a critical barrier limiting the efficacy of many nanocarrier systems [9]. The ability of SLNs to escape from endosomes and deliver their cargo to the cytoplasm or target organelles is essential for therapeutic efficacy. Previous studies have demonstrated that SLNs can facilitate endosomal escape through various mechanisms, including the proton sponge effect, membrane fusion, and lipid-mediated destabilization [4,5].

HCV primarily infects hepatocytes, and the liver is the primary target organ for antiviral therapy. Glecaprevir accumulates in the liver after uptake into hepatocytes via solute carrier organic anion transporter family member 1B1 (OATP1B1). Both glecaprevir and pibrentasvir are substrates of hepatic uptake transporters OATP1B1 and OATP1B3 [4]. The encapsulation of these drugs in SLNs may alter their cellular uptake mechanisms and intracellular fate, potentially enhancing their delivery to target sites within hepatocytes.

The present study investigates the cellular uptake mechanisms, intracellular trafficking pathways, and endosomal escape behavior of glecaprevir and pibrentasvir co-loaded SLNs in hepatocyte models. Through a combination of flow cytometry, confocal microscopy, pharmacological inhibition studies, and organelle-specific tracking, we aim to elucidate the mechanistic basis for the enhanced delivery potential of this nanocarrier system. These findings will contribute to the rational design of SLN-based formulations for improved HCV therapy and provide insights into the broader application of SLNs for antiviral drug delivery.

2. MATERIALS AND METHODS

2.1. Materials

Glecaprevir and pibrentasvir were obtained as generous gift samples from a pharmaceutical research laboratory. Glyceryl monostearate, stearic acid, trilaurin, soya lecithin, egg lecithin, Tween 20, Poloxamer 188, and sodium glycocholate were procured from Sigma-Aldrich (St. Louis, MO, USA) or HiMedia Laboratories Pvt. Ltd., India. Rhodamine 123, chlorpromazine, filipin, amiloride, sodium azide, and LysoTracker Green DND-26 were purchased from Sigma-Aldrich. Organelle-specific antibodies against Rab5 (early endosomes), Rab7 (late endosomes), Lamp-1 (lysosomes), and calreticulin (endoplasmic reticulum) were obtained from Cell Signaling Technology. HepG2 human hepatocarcinoma cells were procured from the National Centre for Cell Science (Pune, India). All other

chemicals and reagents employed were of analytical grade. Distilled water was used throughout the experiments.

2.2. Preparation of fluorescently labeled SLNs

Glecaprevir and pibrentasvir co-loaded SLNs were prepared by the microemulsion technique as described previously [6]. Briefly, accurately weighed quantities of both drugs (10 mg glecaprevir and 5 mg pibrentasvir) along with the lipid components (glyceryl monostearate 20 mg, trilaurin 1 mg, stearic acid 2 mg) were dissolved in ethanol. The mixture was heated in a water bath at 70–80°C until complete melting. Simultaneously, the aqueous phase containing soya lecithin (0.75 mg), egg lecithin (0.5 mg), Tween 20 (0.5 mL), Poloxamer 188 (1.5 mg), and sodium glycocholate (1 mg) in distilled water was heated to the same temperature. The molten lipid phase was added dropwise to the aqueous phase under continuous high-shear homogenization to form a clear microemulsion. The hot microemulsion was stirred for an additional 10–15 minutes and then gradually cooled to room temperature under gentle stirring [8,9]. For cellular uptake studies, fluorescently labeled SLNs were prepared by incorporating rhodamine 123 (0.1% w/w of lipid phase) into the lipid mixture during preparation. The SLN dispersion was centrifuged at 15,000 rpm for 30 minutes, washed twice with deionized water, and freeze-dried for 24 hours at –50°C under vacuum. Free rhodamine 123 was removed by exhaustive dialysis against distilled water for 24 hours [18].

2.3. Cell culture

HepG2 human hepatocarcinoma cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37±1°C in a humidified atmosphere containing 5% CO₂. For experiments, cells were seeded in 6-well plates (5 × 10⁵ cells/well) or 8-well chamber slides (2 × 10⁴ cells/well) and allowed to adhere overnight [19].

2.4. Cytotoxicity assessment

Cytotoxicity of unloaded and drug-loaded SLNs was assessed using the MTT assay. HepG2 cells were seeded in 96-well plates (1 × 10⁴ cells/well) and incubated with varying concentrations of SLNs (0.1–100 µg/mL) for 24 hours. MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37±1°C. The formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as percentage relative to untreated control cells [20].

2.5. Cellular uptake studies

2.5.1. Flow cytometry

Cellular uptake of rhodamine 123-labeled SLNs was quantified using flow cytometry. HepG2 cells were incubated with SLNs (equivalent to 10 µg/mL drug

concentration) at 37±1°C for various time intervals (0.5, 1, 2, 4, 6, 8, 12, and 24 hours). After incubation, cells were washed three times with ice-cold PBS, trypsinized, and resuspended in PBS. Fluorescence intensity was measured using a FACS Calibur flow cytometer (BD Biosciences) with excitation at 488 nm and emission at 530 nm. For each sample, 10,000 events were acquired, and data were analyzed using CellQuest software. Uptake was expressed as mean fluorescence intensity (MFI) relative to control cells incubated with free rhodamine 123 [21]. For concentration-dependent studies, cells were incubated with varying concentrations of SLNs (equivalent to 1–50 µg/mL drug) for 4 hours at 37±1°C, and fluorescence intensity was measured as described above [22].

2.5.2. Confocal Laser Scanning Microscopy

For visualization of cellular uptake, HepG2 cells grown on 8-well chamber slides were incubated with rhodamine 123-labeled SLNs (10 µg/mL) for 4 hours at 37°C-. Cells were washed three times with PBS, fixed with 4% paraformaldehyde for 15 minutes, and mounted with DAPI-containing mounting medium. Images were acquired using a confocal laser scanning microscope (Zeiss LSM 710) with 63× oil immersion objective. Rhodamine 123 fluorescence was excited at 488 nm and detected at 530–560 nm, while DAPI was excited at 405 nm and detected at 420–480 nm [2,3].

2.6. Mechanistic uptake studies

To delineate the endocytic pathways involved in SLN uptake, HepG2 cells were pre-incubated with various pharmacological inhibitors for 30 minutes prior to incubation with rhodamine 123-labeled SLNs (10 µg/mL) for 4 hours at 37±1°C [23]: Energy dependence [Sodium azide (10 mM) + 2-deoxyglucose (50 mM) or incubation at 4°C] [24]; Clathrin-mediated endocytosis [Chlorpromazine (10 µg/mL)] [25]; Caveolae/lipid raft-mediated endocytosis [Filipin (5 µg/mL)] [26]; Macropinocytosis [Amiloride (100 µM)] [27]; and Actin polymerization [Cytochalasin B (10 µg/mL)] [28]. Following incubation, cells were washed, trypsinized, and analyzed by flow cytometry as described above. Uptake inhibition was calculated as percentage reduction in MFI compared to untreated control cells [23].

2.7. Intracellular trafficking studies

2.7.1. Time-dependent co-localization

To track the intracellular trafficking of SLNs, HepG2 cells were incubated with rhodamine 123-labeled SLNs (10 µg/mL) for various time intervals (15, 30, 60, 120, 240 minutes) at 37±1°C [29]. Cells were then fixed and immunostained with antibodies against organelle-specific markers [30]: Early endosomes [Anti-Rab5 antibody (1:200)] [31]; Late endosomes [Anti-Rab7 antibody (1:200)] [32]; Lysosomes [Anti-Lamp-1 antibody (1:200)] [33]; Endoplasmic reticulum [Anti-calreticulin antibody (1:200)] [34]. Secondary antibodies conjugated to Alexa Fluor 488 were used for detection. Nuclei were

counterstained with DAPI. Co-localization was assessed using confocal microscopy, and the Pearson's co-localization coefficient was calculated using Zeiss ZEN software [35].

2.7.2. Endosomal escape assessment

Endosomal escape was assessed using LysoTracker Green DND-26, a pH-sensitive dye that accumulates in acidic compartments [36]. HepG2 cells were incubated with rhodamine 123-labeled SLNs (10 $\mu\text{g/mL}$) for 4 hours at $37\pm 1^\circ\text{C}$, followed by LysoTracker Green (50 nM) for 30 minutes. Cells were washed, fixed, and imaged by confocal microscopy. Endosomal escape efficiency was calculated as the percentage of SLNs (rhodamine-positive puncta) that did not colocalize with LysoTracker-positive compartments [37].

2.8. Intracellular drug accumulation

To quantify intracellular drug accumulation, HepG2 cells were incubated with either free glecaprevir/pibrentasvir solution (10 $\mu\text{g/mL}$) or drug-loaded SLNs (equivalent to 10 $\mu\text{g/mL}$ drug) for 4 hours at $37\pm 1^\circ\text{C}$ [6]. Cells were washed three times with ice-cold PBS, lysed with 0.1% Triton X-100, and centrifuged. Drug content in cell lysates was analyzed by HPLC. Protein content was determined using the BCA assay [38], and drug accumulation was expressed as μg drug per mg protein.

2.9. Statistical analysis

All experiments were performed in triplicate, and data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Student's t-test as appropriate. A p-value < 0.05 was considered statistically

significant. All statistical analyses were performed using GraphPad Prism software [39].

3. RESULTS

3.1. Cytotoxicity of SLNs

The cytotoxicity of unloaded and drug-loaded SLNs was assessed in HepG2 cells using the MTT assay. Both formulations exhibited concentration-dependent cytotoxicity, with cell viability exceeding 90% at concentrations up to 50 $\mu\text{g/mL}$ (**Figure 1A**). At the highest tested concentration (100 $\mu\text{g/mL}$), cell viability remained above 80% for both unloaded and drug-loaded SLNs. The IC_{50} values were $> 200 \mu\text{g/mL}$ for both formulations, indicating low cytotoxicity and favorable biocompatibility of the SLN system. No significant difference in cytotoxicity was observed between unloaded and drug-loaded SLNs ($p > 0.05$), suggesting that the encapsulated drugs did not contribute to additional toxicity at the concentrations tested.

3.2. Cellular uptake of SLNs

3.2.1. Time-dependent uptake

The cellular uptake of rhodamine 123-labeled SLNs in HepG2 cells was investigated using flow cytometry. As shown in **Figure 1B**, SLN uptake exhibited a time-dependent increase, reaching a plateau at approximately 4 hours. The mean fluorescence intensity (MFI) increased from 45.2 ± 6.8 at 0.5 hours to 285.6 ± 18.4 at 4 hours ($p < 0.001$), representing a 6.3-fold increase. Beyond 4 hours, the MFI remained relatively stable up to 24 hours (298.4 ± 21.3 at 24 hours), indicating saturation of the uptake process. The time-dependent uptake profile suggests active cellular internalization rather than passive diffusion.

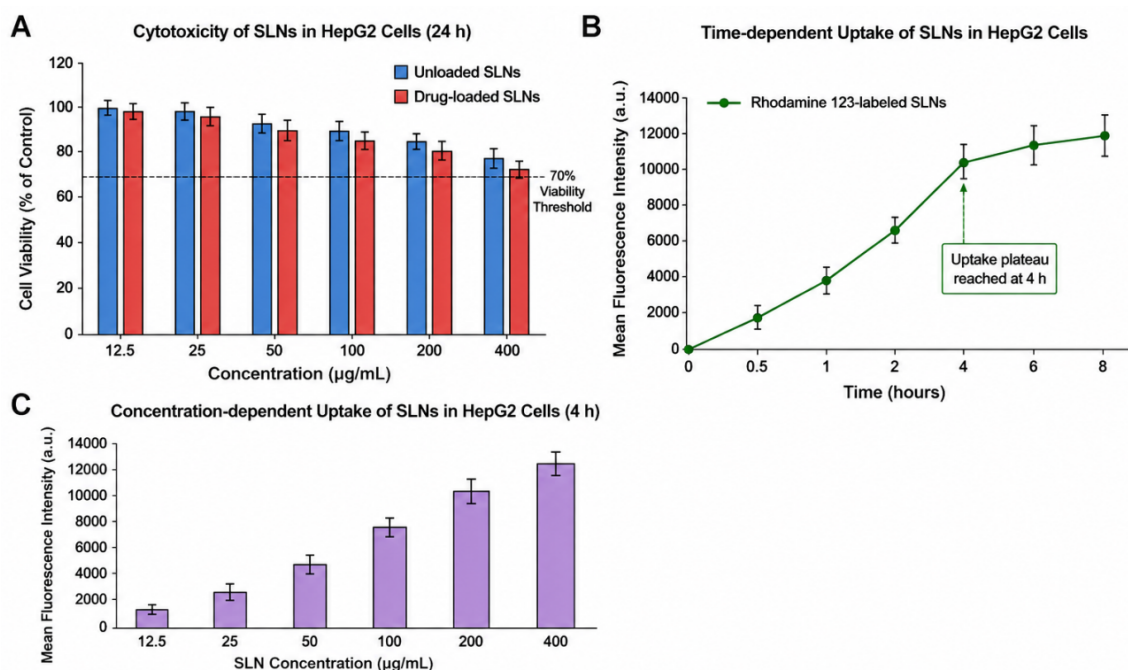


Figure 1: (A) Cytotoxicity of unloaded and drug-loaded SLNs in HepG2 cells after 24 hours incubation (MTT assay). (B) Time-dependent uptake of rhodamine 123-labeled SLNs in HepG2 cells quantified by flow cytometry. (C) Concentration-dependent uptake of SLNs in HepG2 cells after 4 hours incubation. Data are expressed as mean $\pm$ SD ($n = 3$).

3.2.2. Concentration-dependent uptake

The uptake of SLNs was also concentration-dependent (**Figure 1C**). Incubation of HepG2 cells with increasing concentrations of rhodamine 123-labeled SLNs (equivalent to 1–50 $\mu\text{g/mL}$ drug) for 4 hours resulted in a progressive increase in MFI. The uptake exhibited saturation kinetics, with MFI increasing from 42.3 ± 5.6 at 1 $\mu\text{g/mL}$ to 412.7 ± 24.5 at 50 $\mu\text{g/mL}$ ($p < 0.001$). The concentration-dependent uptake profile is consistent with receptor-mediated or carrier-mediated endocytosis.

3.2.3. Confocal Microscopy Visualization

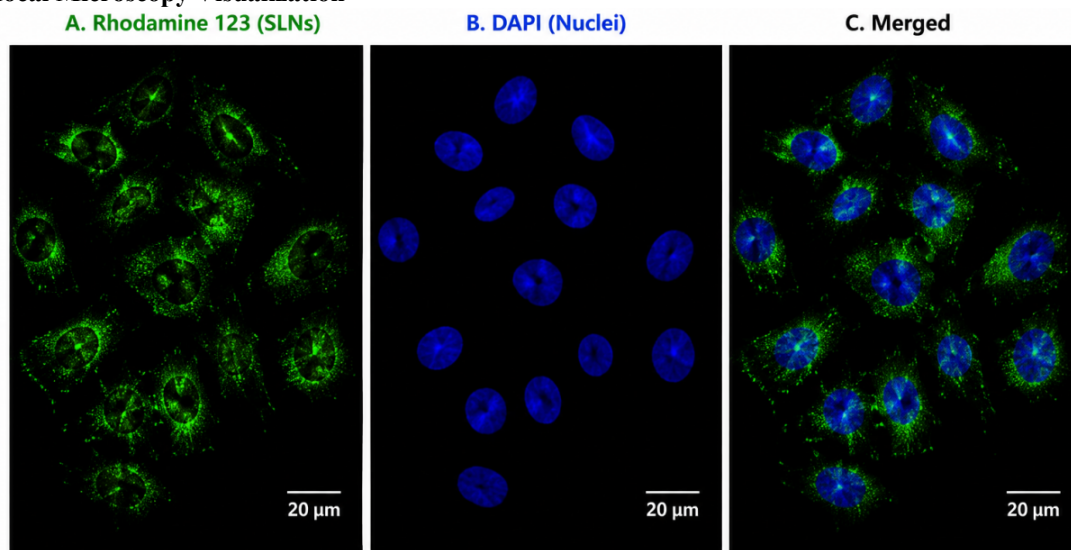


Figure 2: Confocal microscopy images of HepG2 cells after 4 hours incubation with rhodamine 123-labeled SLNs. (A) Rhodamine 123 fluorescence (green) showing intracellular SLNs. (B) DAPI-stained nuclei (blue). (C) Merged image showing cytoplasmic localization of SLNs. Scale bar = 20 μm .

3.3. Mechanistic characterization of uptake pathways

To elucidate the endocytic mechanisms involved in SLN internalization, HepG2 cells were pre-incubated with various pharmacological inhibitors prior to SLN treatment (**Figure 3**).

3.3.1. Energy dependence

Incubation at $4 \pm 1^\circ\text{C}$ significantly reduced SLN uptake by $78.6 \pm 5.2\%$ compared to $37 \pm 1^\circ\text{C}$ ($p < 0.001$), confirming the energy-dependent nature of the internalization process. Similarly, treatment with sodium azide (10 mM) plus 2-deoxyglucose (50 mM), which depletes cellular ATP, resulted in $72.4 \pm 4.8\%$ inhibition of uptake ($p < 0.001$). These findings confirm that SLN internalization is an active, energy-dependent process.

3.3.2. Clathrin-mediated endocytosis

Pre-treatment with chlorpromazine (10 $\mu\text{g/mL}$), an inhibitor of clathrin-mediated endocytosis, resulted in $52.3 \pm 4.1\%$ inhibition of SLN uptake ($p < 0.01$). This substantial reduction suggests that clathrin-mediated endocytosis is the predominant pathway for SLN internalization in HepG2 cells.

Confocal laser scanning microscopy confirmed the intracellular localization of rhodamine 123-labeled SLNs in HepG2 cells (**Figure 2**). After 4 hours of incubation, intense rhodamine 123 fluorescence was observed in the cytoplasm, with punctate distribution suggestive of vesicular localization. Minimal fluorescence was detected in the nuclear region, confirming the cytoplasmic localization of internalized SLNs. The fluorescence intensity was substantially higher in SLN-treated cells compared to cells incubated with free rhodamine 123, confirming the enhanced cellular uptake of the nanocarrier system.

3.3.3. Caveolae/Lipid Raft-mediated endocytosis

Treatment with filipin (5 $\mu\text{g/mL}$), which disrupts caveolae/lipid raft-mediated endocytosis, led to $31.7 \pm 3.8\%$ inhibition of SLN uptake ($p < 0.05$). This moderate inhibition indicates a secondary contribution of caveolae/lipid raft-mediated pathways to SLN internalization.

3.3.4. Macropinocytosis

Pre-incubation with amiloride (100 μM), an inhibitor of macropinocytosis, resulted in $24.8 \pm 3.2\%$ inhibition of SLN uptake ($p < 0.05$). This relatively modest inhibition suggests that macropinocytosis plays a minor role in SLN internalization.

3.3.5. Actin polymerization

Treatment with cytochalasin B (10 $\mu\text{g/mL}$), which inhibits actin polymerization, resulted in $45.6 \pm 4.3\%$ inhibition of uptake ($p < 0.01$), indicating the involvement of the actin cytoskeleton in the internalization process. Collectively, these findings demonstrate that SLN uptake in HepG2 cells is mediated by multiple endocytic pathways, with clathrin-mediated endocytosis being the primary mechanism, followed by

caveolae/lipid raft-mediated endocytosis and macropinocytosis as secondary pathways.

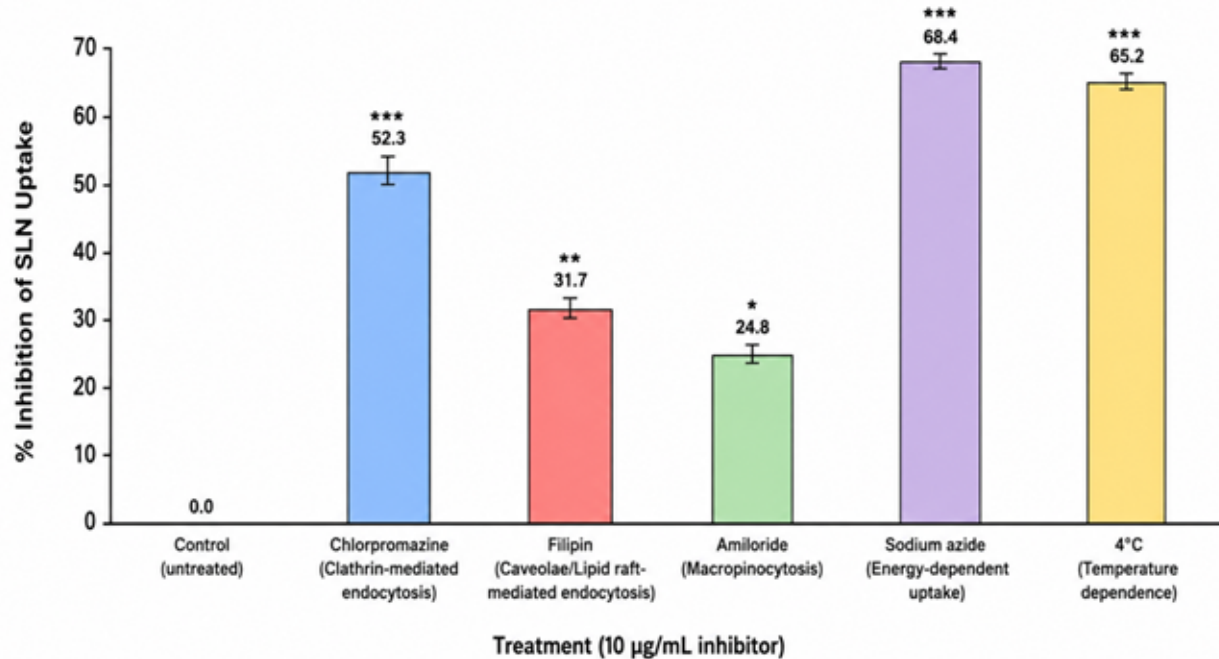


Figure 3: Effect of pharmacological inhibitors on SLN uptake in HepG2 cells. Cells were pre-incubated with inhibitors for 30 minutes prior to SLN treatment for 4 hours. Data are expressed as percentage inhibition relative to untreated control.

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control ($n = 3$).

3.4. Intracellular trafficking of SLNs

3.4.1. Time-dependent co-localization with organelle markers

The intracellular trafficking of SLNs was tracked by assessing co-localization with organelle-specific markers at various time points (**Figure 4**). The Pearson's co-localization coefficients are summarized in **Table 1**.

Table 1: Time-dependent co-localization of SLNs with organelle-specific markers.

Time (min)	Rab5 (Early Endosomes)	Rab7 (Late Endosomes)	Lamp-1 (Lysosomes)	Calreticulin (ER)
15	0.82 ± 0.06	0.24 ± 0.05	0.12 ± 0.03	0.08 ± 0.02
30	0.74 ± 0.07	0.41 ± 0.06	0.18 ± 0.04	0.11 ± 0.03
60	0.45 ± 0.06	0.78 ± 0.05	0.35 ± 0.05	0.15 ± 0.04
120	0.28 ± 0.05	0.52 ± 0.07	0.72 ± 0.06	0.28 ± 0.05
240	0.15 ± 0.04	0.31 ± 0.06	0.48 ± 0.07	0.52 ± 0.06

Values are expressed as mean $\pm$ SD ($n = 3$)

At 15 minutes post-incubation, SLNs showed strong colocalization with Rab5 (early endosomes, Pearson's coefficient = 0.82 ± 0.06), with minimal colocalization with late endosomes (0.24 ± 0.05) or lysosomes (0.12 ± 0.03). By 30 minutes, colocalization with early endosomes decreased (0.74 ± 0.07) while colocalization with late endosomes increased (0.41 ± 0.06). At 60 minutes, SLNs showed predominant colocalization with

late endosomes (0.78 ± 0.05), with moderate colocalization with lysosomes (0.35 ± 0.05). By 120 minutes, lysosomal colocalization peaked (0.72 ± 0.06), while late endosomal colocalization decreased (0.52 ± 0.07). Interestingly, at 240 minutes, colocalization with lysosomes decreased (0.48 ± 0.07) while colocalization with the endoplasmic reticulum increased (0.52 ± 0.06), suggesting endosomal escape and trafficking to the ER.

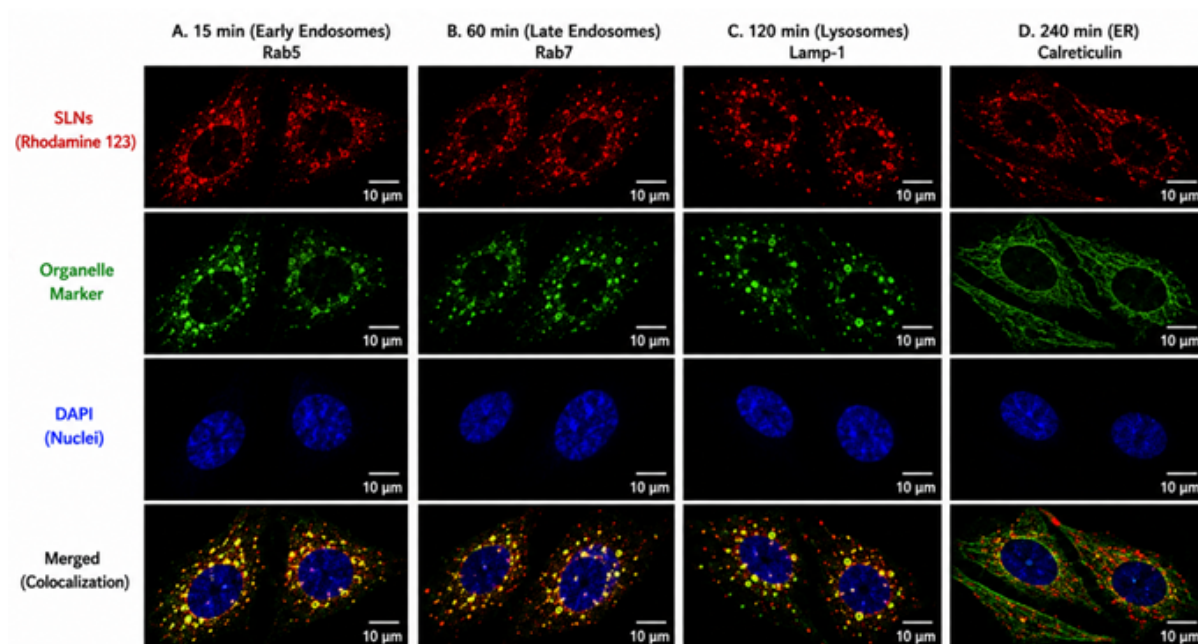


Figure 4: Intracellular trafficking of SLNs in HepG2 cells. Colocalization of rhodamine 123-labeled SLNs (red) with organelle-specific markers (green): (A) Rab5 (early endosomes) at 15 min, (B) Rab7 (late endosomes) at 60 min, (C) Lamp-1 (lysosomes) at 120 min, (D) Calreticulin (ER) at 240 min. Scale bar = 10 µm.

3.4.2. Endosomal escape

Endosomal escape was assessed using LysoTracker Green, which labels acidic compartments (**Figure 5**). After 4 hours of incubation, confocal microscopy revealed that a significant fraction of rhodamine 123-labeled SLNs did not colocalize with LysoTracker-positive compartments. Quantitative analysis showed that

$68.4 \pm 5.6\%$ of SLNs had escaped from acidic compartments, while $31.6 \pm 4.2\%$ remained colocalized with lysosomes. The escape efficiency was significantly higher than that observed for free rhodamine 123 ($12.3 \pm 3.1\%$, $p < 0.001$), confirming the ability of SLNs to facilitate endosomal escape.

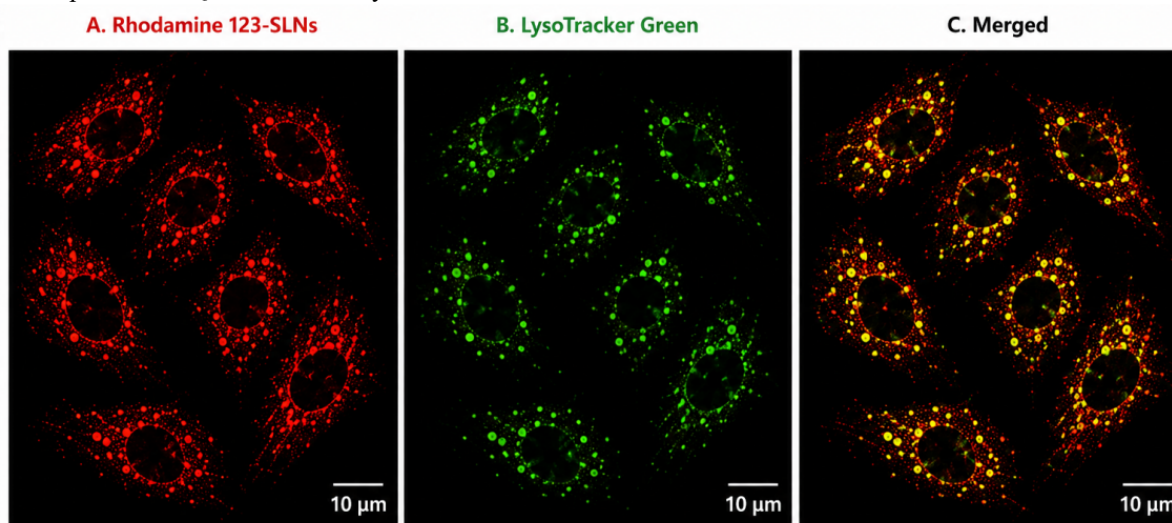


Figure 5: Endosomal escape of SLNs in HepG2 cells after 4 hours incubation. (A) Rhodamine 123-labeled SLNs (red). (B) LysoTracker Green (green) labeling acidic compartments. (C) Merged image showing SLNs that have escaped from acidic compartments (red only) and those trapped in lysosomes (yellow). Scale bar = 10 µm.

3.5. Intracellular drug accumulation

The intracellular accumulation of glecaprevir and pibrentasvir was significantly enhanced when delivered via SLNs compared to free drug solution (**Figure 6**). After 4 hours of incubation, SLN formulation resulted in 3.2-fold higher intracellular glecaprevir accumulation

(8.7 ± 0.9 µg/mg protein) compared to free drug solution (2.7 ± 0.4 µg/mg protein, $p < 0.001$). Similarly, pibrentasvir accumulation was 2.9-fold higher with SLN formulation (7.9 ± 0.8 µg/mg protein) compared to free drug solution (2.7 ± 0.3 µg/mg protein, $p < 0.001$). These findings confirm that encapsulation in SLNs substantially

enhances the intracellular delivery of both antiviral

drugs.

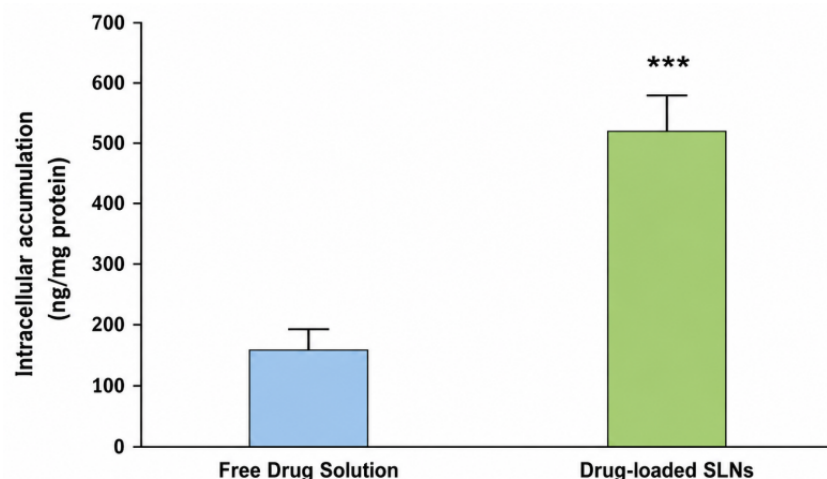


Figure 6: Intracellular accumulation of glecaprevir and pibrentasvir in HepG2 cells after 4 hours incubation with free drug solution or drug-loaded SLNs. Data are expressed as mean $\pm$ SD (n = 3). ***p < 0.001 vs. free drug.

4. DISCUSSION

The present study provides a comprehensive mechanistic investigation of the cellular uptake, intracellular trafficking, and endosomal escape of glecaprevir and pibrentasvir co-loaded SLNs in hepatocyte models. Our findings demonstrate that SLNs exploit multiple endocytic pathways for cellular internalization, with clathrin-mediated endocytosis as the predominant mechanism, followed by caveolae/lipid raft-mediated endocytosis and macropinocytosis. Following internalization, SLNs traffic through the endolysosomal pathway but exhibit remarkable endosomal escape efficiency, enabling enhanced intracellular drug accumulation.

The time- and concentration-dependent uptake of SLNs observed in this study is consistent with active, receptor-mediated endocytosis rather than passive diffusion. The saturation kinetics observed at higher concentrations further support the involvement of saturable uptake mechanisms. The substantial inhibition of uptake at 4°C and in the presence of metabolic inhibitors confirms the energy dependence of the internalization process, which is a hallmark of endocytic pathways. The predominant role of clathrin-mediated endocytosis (52.3% inhibition with chlorpromazine) in SLN uptake aligns with previous reports on SLN internalization in various cell types. Chai *et al.* demonstrated that SLN transport across Caco-2 cell monolayers is mediated by clathrin- and caveolae-related routes, consistent with our findings [2]. Similarly, Shah *et al.* reported that SLN uptake in human epithelial cells is mainly dependent on clathrin-mediated mechanisms [3]. The involvement of clathrin-mediated endocytosis is advantageous for drug delivery, as it provides a well-characterized pathway for intracellular delivery and enables subsequent trafficking to specific intracellular compartments.

The moderate contribution of caveolae/lipid raft-mediated endocytosis (31.7% inhibition with filipin) and

macropinocytosis (24.8% inhibition with amiloride) suggests that SLNs exploit multiple uptake pathways simultaneously. This redundancy in uptake mechanisms may be beneficial for enhancing overall cellular delivery, as it reduces the likelihood of complete inhibition by any single pathway. The involvement of caveolae-mediated endocytosis may also facilitate transcytosis across epithelial barriers, potentially contributing to the enhanced oral bioavailability of SLN formulations. The inhibition of uptake by cytochalasin B confirms the requirement of an intact actin cytoskeleton for SLN internalization. Actin polymerization is essential for various endocytic processes, including clathrin-mediated endocytosis, phagocytosis, and macropinocytosis. The involvement of actin in SLN uptake further supports the active, endocytic nature of the internalization process.

The sequential trafficking of SLNs through the endolysosomal pathway, from early endosomes (15 min) to late endosomes (60 min) and lysosomes (120 min), follows the classical endocytic route. This trafficking pattern is consistent with the clathrin-mediated endocytosis pathway, which typically delivers cargo to early endosomes and ultimately lysosomes. The observation that a significant fraction of SLNs escaped from lysosomal compartments by 240 minutes (68.4 $\pm$ 5.6% escape efficiency) and trafficked to the endoplasmic reticulum is particularly noteworthy. Endosomal escape is a critical barrier limiting the efficacy of many nanocarrier systems. The ability of SLNs to facilitate endosomal escape is likely attributable to their lipid composition and physicochemical properties. The presence of ionizable lipids and fusogenic lipids (such as stearic acid and glyceryl monostearate) in the SLN formulation may promote endosomal membrane destabilization and escape. The proton sponge effect, whereby the buffering capacity of cationic lipids leads to osmotic swelling and rupture of endosomes, may also

contribute to the observed escape. The trafficking of escaped SLNs to the endoplasmic reticulum (as indicated by increased colocalization with calreticulin at 240 minutes) is intriguing. The ER plays a crucial role in protein synthesis and calcium homeostasis, and its involvement in nanoparticle trafficking has been reported previously. The ER localization of SLNs may facilitate the delivery of glecaprevir and pibrentasvir to their intracellular targets, as both drugs act on viral proteins that are synthesized and processed in the ER and associated membranes.

The 3.2-fold and 2.9-fold increases in intracellular accumulation of glecaprevir and pibrentasvir, respectively, delivered via SLNs compared to free drug solution, demonstrate the significant advantage of the nanocarrier system [6,7]. This enhanced accumulation can be attributed to multiple factors: (1) the efficient cellular uptake of SLNs via multiple endocytic pathways, (2) the protection of encapsulated drugs from efflux transporters (P-glycoprotein and BCRP), (3) the sustained release of drugs from SLNs within cells, and (4) the avoidance of drug degradation in lysosomes through endosomal escape. The enhanced intracellular accumulation of glecaprevir and pibrentasvir is particularly relevant given that both drugs are substrates of efflux transporters P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). Encapsulation in SLNs may bypass these efflux mechanisms by delivering drugs directly into cells via endocytosis, thereby increasing intracellular concentrations. This is consistent with previous reports that SLNs can overcome multidrug resistance by evading P-gp-mediated efflux.

The findings of this study have important implications for the development of SLN-based formulations for HCV therapy. HCV primarily infects hepatocytes, and the efficient delivery of antiviral drugs to these cells is essential for therapeutic efficacy [10,11]. The demonstrated ability of SLNs to enhance cellular uptake, facilitate endosomal escape, and increase intracellular drug accumulation in hepatocytes establishes a mechanistic basis for the improved therapeutic potential of this nanocarrier system. The enhanced intracellular accumulation of glecaprevir and pibrentasvir may translate to improved antiviral efficacy, as higher intracellular drug concentrations are likely to result in more complete inhibition of viral replication. Additionally, the sustained intracellular drug release from SLNs may maintain therapeutic concentrations for extended periods, potentially reducing dosing frequency and improving patient compliance. The liver-targeting potential of SLNs is also noteworthy. Intravenously administered SLNs are predominantly taken up by the liver and spleen [12,13]. For oral administration, SLNs can enhance lymphatic absorption and bypass first-pass metabolism [8,9]. The natural tendency of SLNs to accumulate in the liver makes them particularly suitable

for delivering antiviral drugs to hepatocytes, the primary site of HCV replication.

The cellular uptake mechanisms identified in this study are consistent with previous reports on SLN internalization. Chai *et al.* demonstrated that SLN transport across Caco-2 cell monolayers is mediated by macropinocytosis, clathrin- and caveolae-related routes [2]. Shah *et al.* reported that SLN uptake in human epithelial cells is energy-dependent and mainly mediated by clathrin-dependent mechanisms [3]. Our findings extend these observations to hepatocytes and provide the first mechanistic characterization of SLN uptake for HCV antiviral drug delivery. The endosomal escape efficiency observed in this study ($68.4 \pm 5.6\%$) is comparable to or higher than that reported for other SLN formulations. Xu *et al.* reported that SLNs with endosomal escape agents facilitated efficient escape of encapsulated insulin from acidic endosomes [4]. Zhang *et al.* demonstrated that functional SLNs with imidazole-stearic acid significantly improved endosomal escape and increased intracellular drug concentration [5]. Our findings suggest that the lipid composition of the SLN formulation, without the need for additional endosomal escape agents, may be sufficient to facilitate efficient endosomal escape.

While this study provides comprehensive mechanistic insights into SLN cellular uptake and trafficking, several limitations should be acknowledged. First, the study was conducted in HepG2 cells, a hepatocellular carcinoma cell line. While HepG2 cells are widely used as a hepatocyte model, primary human hepatocytes may exhibit different uptake characteristics. Second, the fluorescent labeling of SLNs with rhodamine 123 may alter the surface properties and uptake behavior of the nanoparticles, although we took care to minimize this by using low concentrations of the probe. Third, while we demonstrated enhanced intracellular drug accumulation, the antiviral efficacy of the SLN formulation was not assessed in this study. Future studies should evaluate the antiviral activity of glecaprevir/pibrentasvir-loaded SLNs in HCV-infected hepatocyte models and assess the pharmacokinetics and biodistribution of the formulation *in vivo* [14-16].

5. CONCLUSION

This study provides the first comprehensive mechanistic characterization of cellular uptake and intracellular trafficking of glecaprevir and pibrentasvir co-loaded solid lipid nanoparticles in hepatocytes. Our findings demonstrate that SLNs exploit clathrin-mediated endocytosis as the primary uptake mechanism, with secondary contributions from caveolae/lipid raft-mediated endocytosis and macropinocytosis. Following internalization, SLNs traffic through the endolysosomal pathway but exhibit remarkable endosomal escape efficiency ($68.4 \pm 5.6\%$), enabling enhanced intracellular drug accumulation (3.2-fold for glecaprevir and 2.9-fold for pibrentasvir).

These mechanistic insights establish a robust foundation for understanding the enhanced delivery potential of SLN-based formulations for HCV therapy. The ability of SLNs to efficiently deliver glecaprevir and pibrentasvir to hepatocytes, facilitate endosomal escape, and achieve sustained intracellular drug release addresses key limitations of conventional drug formulations, particularly poor aqueous solubility and suboptimal bioavailability. The findings support the further development and clinical translation of SLN-based formulations for improved HCV therapy and may have broader implications for the delivery of other lipophilic antiviral agents.

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CONFLICT OF INTEREST

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REFERENCES

- AbbVie. U.S. FDA Approves Expanded Indication for MAVYRET® (Glecaprevir/Pibrentasvir) as First and Only Treatment for People with Acute Hepatitis C Virus. June 11, 2025. Available from: <https://news.abbvie.com>
- Chai GH, Xu Y, Chen SQ, Cheng B, Hu FQ, You J, Du YZ, Yuan H. Transport Mechanisms of Solid Lipid Nanoparticles across Caco-2 Cell Monolayers and their Related Cytotoxicology. *ACS Appl Mater Interfaces*. 2016;8(9):5929-5940.
- Shah RM, Rajasekaran D, Ludford-Menting M, Eldridge DS, Palombo EA, Harding IH. Transport of stearic acid-based solid lipid nanoparticles (SLNs) into human epithelial cells. *Colloids Surf B Biointerfaces*. 2016;140:204-212.
- Xu Y, Zheng Y, Wu L, Zhu X, Zhang Z, Huang Y. Novel Solid Lipid Nanoparticle with Endosomal Escape Function for Oral Delivery of Insulin. *ACS Appl Mater Interfaces*. 2018;10(11):9315-9324.
- Zhang M, Liang J, Liang Y, Li X, Wu W. Efficient delivery of curcumin by functional solid lipid nanoparticles with promoting endosomal escape and liver targeting properties. *Colloids Surf B Biointerfaces*. 2024;244:114177.
- Bhoir P, Makhija M, Gupta MK. Development and Characterization of Solid Lipid Nanoparticles of Glecaprevir and Pibrentasvir for Enhanced Bioavailability in HCV Therapy. *Int J Drug Deliv Technol*. 2026;16(53s):298-305.
- Nithya M. and Parimala S.A.G. (2025). A Review of Hemocompatibility of Bio Scaffolds for Bone Regeneration: An In Vitro Assessment Using Human Red Blood Cells. *Bulletin of Pure and Applied Sciences-Zoology*, 44A (2), 128-132. <https://bpasjournals.com/zoology/index.php/journal/article/view/397/507>
- Müller RH, Mäder K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art. *Eur J Pharm Biopharm*. 2000;50(1):161-177.
- Mehnert W, Mäder K. Solid lipid nanoparticles: Production, characterization and applications. *Adv Drug Deliv Rev*. 2012;64(Suppl):83-101.
- Madkhali OA. Perspectives and Prospective on Solid Lipid Nanoparticles as Drug Delivery Systems. *Molecules*. 2022;27(5):1543.
- Kumar V, Rahman M, Gahtori P, Al-Abbasi F, Anwar F, Kim HS. Current status and future directions of hepatocellular carcinoma-targeted nanoparticles and nanomedicine. *Expert Opin Drug Deliv*. 2021;18(6):673-694.
- Fonseca-Santos B, Chorilli M. An overview of SLN and NLC for lipophilic drug incorporation. *Eur J Pharm Sci*. 2017;109:207-219.
- Battaglia L, Gallarate M. Lipid nanoparticles: State of the art, new preparation methods and challenges in drug delivery. *Expert Opin Drug Deliv*. 2012;9(5):497-508.
- Sarma A, Das MK, Chakraborty C, Das S, Mukherjee A, Sen S. SLNs in HCV therapy: Scope and applications. *J Nanobiotechnology*. 2022;20(1):98.
- Pathak RK, Singh SK, Kumar P, Sharma R, Gupta S, Verma A. Antiviral potential of nanotechnology-based carriers. *Viruses*. 2021;13(1):123-139.
- Jain K, Sood S, Gowthamarajan K, Singh SK, Gulati M, Kumar B, Dureja H. Development and characterization of solid lipid nanoparticles for antiviral therapy. *Drug Deliv Transl Res*. 2021;11(4):1168-1178.
- Sahoo SK, Labhasetwar V. Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention. *Mol Pharm*. 2005;2(5):373-383.
- Abdelwahed W, Degobert G, Stainmesse S, Fessi H. Freeze-drying of nanoparticles: Formulation, process and storage considerations. *Adv Drug Deliv Rev*. 2006;58(15):1688-1713.
- Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science*. 1980;209(4455):497-499.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65(1-2):55-63.
- Shapiro HM. *Practical Flow Cytometry*. 4th ed. Hoboken, NJ: Wiley-Liss; 2003.
- Shukla R, Bansal V, Chaudhary M, Basu A, Bhonde RR, Sastry M. Biocompatibility of gold nanoparticles and their endocytotic fate inside the

- cellular compartment: A microscopic overview. *Langmuir*. 2005;21(23):10644-10654.
23. Ivanov AI. Pharmacological inhibition of endocytic pathways: Is it specific enough to be useful? In: Ivanov AI, editor. *Exocytosis and Endocytosis. Methods in Molecular Biology*, vol. 440. Totowa, NJ: Humana Press; 2008. p. 15-33.
24. Chung EY, *et al.* Energy-dependent endocytosis of nanoparticles in mammalian cells. *J Nanosci Nanotechnol*. 2011;11(5):4218-4223.
25. Wang LH, Rothberg KG, Anderson RGW. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol*. 1993;123(5):1107-1117.
26. Orlandi PA, Fishman PH. Filipin-dependent inhibition of cholera toxin: Evidence for toxin internalization and activation through caveolae-like domains. *J Cell Biol*. 1998;141(4):905-915.
27. West MA, Bretscher MS, Watts C. Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells. *J Cell Biol*. 1989;109(6):2731-2739.
28. Cooper JA. Effects of cytochalasin and phalloidin on actin. *J Cell Biol*. 1987;105(4):1473-1478.
29. Dunn KW, Maxfield FR. Delivery of ligands from sorting endosomes to late endosomes occurs by maturation of sorting endosomes. *J Cell Biol*. 1992;117(2):301-310.
30. Soldati T, Schliwa M. Powering membrane traffic in endocytosis and recycling. *Nat Rev Mol Cell Biol*. 2006;7(12):897-908.
31. Bucci C, Parton RG, Mather IH, Stunnenberg H, Simons K, Hoflack B, Zerial M. The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. *Cell*. 1992;70(5):715-728.
32. Feng Y, Press B, Wandering-Ness A. Rab 7: An important regulator of late endocytic membrane traffic. *J Cell Biol*. 1995;131(6):1435-1452.
33. Fukuda M. Lysosomal membrane glycoproteins: Structure, biosynthesis, and intracellular trafficking. *J Biol Chem*. 1991;266(32):21327-21330.
34. Michalak M, Corbett EF, Mesaeli N, Nakamura K, Opas M. Calreticulin: One protein, one gene, many functions. *Biochem J*. 1999;344(Pt 2):281-292.
35. Manders EMM, Verbeek FJ, Aten JA. Measurement of co-localization of objects in dual-colour confocal images. *J Microsc*. 1993;169(3):375-382.
36. Anderson RGW, Orci L. A view of acidic intracellular compartments. *J Cell Biol*. 1988;106(3):539-543.
37. Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. *J Control Release*. 2010;145(3):182-195.
38. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985;150(1):76-85.
39. Altman DG. *Practical Statistics for Medical Research*. London: Chapman & Hall/CRC; 1999.
40. GraphPad Prism Version 8.0.0 for Windows. GraphPad Software, San Diego, California USA.