Ameliorating the antitumor activity of gemcitabine against breast tumor using \( \alpha_{\nu}\beta_3 \) integrin-targeting lipid nanoparticles

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ABSTRACT

Objective: The main objective is to formulate solid lipid nanoparticles conjugated with cyclic RGDfK peptide encapsulated with gemcitabine hydrochloride drug for targeting breast cancer.

Significance: The hydrophilic nature of gemcitabine hampers passive transport by cell membrane permeation that may lead to drug resistance as it has to enter the cells via nucleoside transporters. The art of encapsulating the drug in a nanovesicle and then anchoring it with a targeting ligand is one of the present areas of research in cancer chemotherapy.

Methods: In this study, solid lipid nanoparticles were prepared by double emulsification and solvent evaporation method. Cyclic RGDfK and gemcitabine hydrochloride were used as targeting ligands and chemotherapeutic drugs, respectively, for targeting breast cancer. The prepared nanoparticles were evaluated for in vitro and in vivo performance to showcase the targeting efficiency and therapeutic benefits of the gemcitabine-loaded ligand conjugated nanoparticles.

Results: When compared with gemcitabine (GEM) and GEM loaded nanoparticles (GSLN), the ligand conjugated GEM nanoparticles (cGSLN) showed superior cytotoxicity, apoptosis, and inhibition of 3D multicellular spheroids in human breast cancer cells (MDA MB 231). The in vivo tumor regression studies in orthotopic breast cancer induced Balb/c mice showed that cGSLN displayed superior tumor suppression and also the targeting potential of the cGSLN toward induced breast cancer.

Conclusion: Prepared nanoformulations showed enhanced anticancer activity in both 2D and 3D cell culture models along with antitumor efficacy in orthotopic breast cancer mouse models.

GRAPHICAL ABSTRACT
**Introduction**

As per GLOBOCAN 2020 estimates, female breast cancer has now excelled lung cancer as the most widely distributed cancer worldwide. Among women, one in four cancer cases and one in six cancer deaths were accounted for this cancer as stated by the International Agency for Research on Cancer (IARC). It has now taken the first position as the primary cause of global cancer incidence in 2020, accounting for about 2.3 million new cases (11.7%), followed by lung at 11.4%. Further, female breast cancer is the fifth leading cause of death globally with 685,000 deaths which are about 6.9% of total deaths [1].

Presently available treatments for breast cancer depend on the disease stage and are radiotherapy, surgery, chemotherapy, hormone therapy, etc. All of them have their own drawbacks and are not efficient enough to eradicate cancer. These limitations of the established methods have imposed to develop targeted therapy which is intended at reducing the side effects caused by means of anticancer drugs and is also expected at drawing maximum therapeutic efficacy at minimum doses to avoid undesirable off-target side effects [2–7]. Globally, many researchers are working tirelessly toward the development of an efficient nanocarrier system that targets specifically cancer [8–10].

In this present research, solid lipid nanoparticles (SLN) were prepared with encapsulated gemcitabine hydrochloride and are conjugated with a targeting pentapeptide- cyclic(Arg-Gly-Asp-D-Phe-Lys) (cRGDfK) for directing the nanoparticles toward breast cancer cells which harbor \( \alpha_\text{IIb} \beta_\text{3} \) integrin receptors [11]. Both cyclic and linear pentapeptides have gained popularity because of their specific targeting and ability to deliver cargos to cancerous cells [12]. Integins are a group of cell adhesion receptors that are highly present on endothelial cells and tumor cells however, are scantily present in resting endothelial and normal cells [11]. Thus, they are used as a target for delivering the anticancer drug.

Gemcitabine hydrochloride (GEM) is a highly effective anticancer agent and has been clinically approved for the treatment of different cancers like breast, non-small cell lung, bladder, ovarian and pancreatic [13–16]. However, utilization of GEM as traditional chemotherapy poses many drawbacks like it undergoes fast metabolism with a very tiny half-life of < 20 min which leads to non-uniform distribution in tissues [17–19]. The hydrophilic nature of GEM impedes the passive transport that may lead to drug resistance as GEM enters the cells via nucleoside transporters. It also shows non-specificity by infusing into both normal and cancerous cells. Hence, to avoid these unwanted effects and improving its anticancer efficacy, it needs to be encapsulated into a biocompatible nanocarrier.

In this research work, solid lipid nanoparticles were hired for loading the GEM as they are well-established delivery systems to encapsulate both hydrophilic and hydrophobic drugs with good stability. Their surface can be easily functionalized as they can participate in conjugation with various types of targeting ligands. Thus, the overall objective of this research work, i.e. improving the anticancer efficacy of the GEM can be accomplished by targeting it to integrin receptors via cRGDfK peptide.

**Materials and methods**

**Materials**

Glyceryl monostearate (GMS) was procured from Alfa Aesar (Johnson Matthey Chemicals, Hyderabad, India). Stearic acid, dialysis tubing (molecular weight (MW) cut off 12,000–14,000 Da), thiazolyl blue tetrazolium bromide (MTT), \( N^-\text{ethylcarbodiimide} \) (EDC), \( N^-\text{hydroxysuccinimide} \) (NHS), dimethyl sulfoxide (DMSO), annexin V-FITC apoptosis detection kit, D-\( \alpha^-\text{tocopheryl polyethylene glycol succinate} \) (TPGS), 2',7'-Dichlorofluorescin diacetate (DCFDA), coumarin 6 (C6), 5,5',6,6'-Tetrachloro-1',3',3'-tetraethyl-imidacarbocyanine iodide (JC-1) were obtained from Sigma–Aldrich (St., Louis, MO, USA). Lechitin soy was acquired from Himedia (Mumbai, India). Gemcitabine hydrochloride (GEM) was a kind gift from TherDose Pharma Pvt. Ltd. (Hyderabad, India). cRGDfK was purchased from Peptide International (Kentucky, USA). High-performance liquid chromatography (HPLC) grade solvents, such as chloroform, methanol, and acetonitrile were purchased from Merck Limited (Mumbai, India). MDA MB 231 human breast cancer cells and 4T1 murine mammary carcinoma cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Dulbecco’s modified eagle’s media (DMEM), trypsin-EDTA, fetal bovine serum (FBS), and phosphate buffer saline (PBS) were bought from Gibco, USA. Hoechst 33342 were procured from ThermoFischer Scientific (Caribbean, Australia). Ultra-low attachment (spheroid) 96-well plates were obtained from Corning (Mulgrave, Australia). Tween 80 was a product of SD Fine Chem Ltd. (Mumbai, India). All other remaining chemicals and solvents were of analytical grade and used further without any purification.

**Optimization and preparation of solid lipid nanoparticles (SLN)**

Solid lipid nanoparticles were prepared by double emulsification and solvent evaporation method [20,21], which involves the formation of multiple emulsions of w/o/w type. In the initial step, known as primary emulsification w/o emulsion was made where 200 \( \mu \)L of aqueous phase having GEM was added to oily (choloroform) phase containing lipids like glycercyl monostearate (80 mg), stearic acid (20 mg) and soya lecithin (30 mg) using sonication energy by employing probe sonicator (Vibra Cell, Sonics, USA) for 5 min. This prepared primary emulsion was subsequently added to the outer aqueous phase containing surfactant to shape the secondary emulsion (w/o/w) by using a sonication process for 7 min. This is finally added to the aqueous phase and kept for solvent evaporation for the hardening and formation of nanoparticles. The resulting GEM-loaded SLN (GSLN) were centrifuged for 40 min at 13,000 rpm. The supernatant was analyzed for calculating the free drug resistance as it enters the cells via nucleoside transporters. It also shows non-specificity by infusing into both normal and cancerous cells. Hence, to avoid these unwanted effects and improving its anticancer efficacy, it needs to be encapsulated into a biocompatible nanocarrier.

In this research work, solid lipid nanoparticles were prepared for loading the GEM as they are well-established delivery systems to encapsulate both hydrophilic and hydrophobic drugs with good stability. Their surface can be easily functionalized as they can participate in conjugation with various types of targeting ligands. Thus, the overall objective of this research work, i.e. improving the anticancer efficacy of the GEM can be accomplished by targeting it to integrin receptors via cRGDfK peptide.

**Optimization for higher encapsulation efficiency**

To enhance the encapsulation efficiency of the hydrophilic drug, various electrolytes were added into the external aqueous phase to avoid leakage of it in the external aqueous phase. Electrolytes like calcium chloride and sodium chloride were used here. In addition, the incorporation of surfactants in the internal aqueous
phase also played a crucial role in increasing the encapsulation efficiency. Tween 80 and TPGS were selected as internal surfactants to observe their effects in increasing the encapsulation efficiency [22].

Encapsulation efficiency (EE) of GEM into nanoparticles was calculated by utilizing an HPLC system (Waters, USA). Supernatant containing free drug was utilized to determine the amount of drug encapsulated in the nanoparticles. The percentage of encapsulated GEM was determined by using the formula, % EE = (1 - (As/Al)) × 100, where As: amount of drug present in supernatant; Al: initial amount of drug added to prepare the GEM loaded SLN.

**Analytical method development for estimation of GEM by HPLC**

The drug GEM was quantified using an HPLC system in reverse phase (Waters, USA) having a photodiode array detector and C18 column (Waters Reliant, 5 µm, 4.6 × 250 mm). A combination of acetonitrile and water (25:75, v/v) was employed as a solvent mixture with a flow rate of 0.7 ml/min, and peaks were examined at 268 nm as detection wavelength. Variations in the intra and inter-day sample analysis were within the limits with relative standard deviation <5%. The calibration graph was plotted using the area under curve (AUC) for respective concentrations by linear regression analysis with the equation \( y = mx + c \) where \( y \) is AUC generated by GEM, \( x \) is concentration of GEM, and \( m \) and \( c \) are slope and intercept, respectively.

**Surface coupling of cRGDK peptide**

cRGDK peptide was conjugated to the surface of nanoparticles by a well-versed carbodiimide reaction, in which 25 mg of GSLN nanoparticles were taken in 5 ml of phosphate buffer saline (PBS) of pH 7.4 and were incubated in EDC and NHS to activate the carboxyl groups on the surface of nanoparticles for an hour at environment with the equation of pH 7.4 and were incubated in EDC and NHS to activate the carboxyl groups on the surface of nanoparticles. The percentage of encapsulated GEM was determined by using the formula, % EE = (1 – (As/Al)) × 100, where As: amount of drug present in supernatant; Al: initial amount of drug added to prepare the GEM loaded SLN.

**Characterization of the conjugated nanoparticles**

The conjugation between cRGDK and carboxylic group of SLN lead to the formation of cRGDK grafted nanoparticles which was confirmed using an FTIR spectrophotometer (Perkin Elmer, UK). Samples were converted into pellets by mixing with potassium bromide salt and compressed using a hydraulic press. The transmittance for pellets was obtained by scanning at a range of 400–4000 cm⁻¹ wavenumber.

**Characterization of nanoparticles**

Dynamic light scattering (DLS) was employed to find out the particle size, polydispersity index (PDI), and zeta potential for BSLN, GSLN, and cGSLN using zetasizer Nano-ZS (Malvern Instrument Ltd., UK). To verify the physical state of GEM in pure form and after loading into lipid nanoparticles, differential scanning calorimetry (DSC) analysis was done by employing DSC STAR ONE (Mettler, Switzerland). Pure GEM and GSLN each 5 mg were weighed and sealed into the aluminum pan. Thermograms were then obtained by running the samples at a temperature increment of 10°C/min, and scanning range of 30–350°C under the influence of nitrogen. FTIR of GEM, lyophilized GSLN and cGSLN was carried out by FTIR spectrometer. In addition the morphology of the prepared nanoparticles cGSLN was performed by TEM (transmission electron microscope) analysis, where a drop of sample was placed on a carbon-coated copper grid, dried and observed.

**In vitro drug release**

The dialysis bag method (dialysis membrane with MW 12,000–14,000 Da) was utilized to find out the release performance of GEM encapsulated nanoparticles viz. GSLN and cGSLN which were then judged against pure GEM [13]. Release media employed for this purpose were sodium acetate buffer (SAB) of pH 5.5 and PBS with pH 7.4 which mimics the pH of the tumor microenvironment and physiological fluid, respectively. All samples viz. GEM, GSLN, and cGSLN, were taken at an equivalent of 1 mg of GEM and were dispersed in 0.5% Tween 80 solution, filled in dialysis bags, tightly tied at both the ends to prevent leakage, and kept in 200 ml of drug release media at an ambient temperature of 37 ± 0.5°C, and stirred at 100 rpm. At particular time points, one mL of samples were taken out from respective media and replaced with suitable buffers to maintain the sink conditions. The collected samples of various time points were analyzed using an HPLC instrument for estimation of the GEM in vitro release profile.

**Steric stability against electrolyte-induced aggregation**

The steric stability of the prepared nanoparticles was determined by employing the solution containing flocculating agent sodium sulfate (Na₂SO₄) dissolved in 16.7% w/w sucrose was utilized. Prepared solid lipid nanoparticles were dispersed in a flocculating solution of various molarities like 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 1.4, 2.0 M. After the addition of SLN to the flocculating mixture, immediately the turbidity of them was measured using a plate reader (Synergy-4, Biotek, USA) at 600 nm [24].

**Colloidal stability**

The change in physicochemical parameters like particle size, and zeta potential in different solutions like normal saline (NS), PBS, plasma, and distilled water with time gives an idea of the colloidal stability of SLN. Aliquots of the stock solution of nanoparticles (1 mg/mL) were then dispersed in the above said media. At specified time points, samples were analyzed for the change in particle size and zeta potential using DLS, a Zetasizer Nano-ZS.

**Cell culture studies**

**Anti-proliferation assay**

MDA MB 231 human breast cancer cells were seeded at 4 × 10⁴ cells/well in 96 well plates. After incubating for 24 h the adhered cells were used to check the half-maximal inhibitory concentration (IC₅₀) of the formulations along with the pure GEM which was treated at different concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5 µM and incubated for 48 h. MTT (5 mg/mL) was then added at a volume of 10 µL per well and placed in an incubator maintained at 37°C and 5% CO₂ for 4 h, after which the media was then substituted with dimethyl sulfoxide which leads to the dissolution of the formazan crystals and absorbance was read at 570 nm using multidetection microplate reader (Synergy-4, Biotek,
For the calculation of IC₅₀, untreated cells were employed as control cells [25].

**Cellular uptake study**

**Quantitative through FACS.** Intracellular trafficking of the lipid nanoparticles was performed by using a fluorescent dye named coumarin-6 (C6) loaded SLN (C6 SLN). For the preparation of C6 SLN, the drug GEM was replaced with C6, where C6 was dissolved along with lipids in the organic phase and used in the preparation of the SLN by double emulsification and solvent evaporation method. For the preparation of cRGDK conjugated C6 SLN, i.e., C6 cSLN, the lyophilized powder of C6 SLN was used and the same method was followed as in the preparation of cGSLN. About 1 × 10⁶ MDA MB 231 cells were added to 6 well plates and allowed to adhere in the incubator. They were then treated with media containing C6 alone, C6 solid lipid nanoparticles, and C6 cSLN conjugated lipid nanoparticles at 5 μg/mL concentration. After 24h exposure, the cells were centrifuged at 4°C and 1200 rpm for 5 min, washed thrice with PBS to remove unentered nanoparticles and excess free dye. The cells were redispersed in PBS and analyzed by flow cytometry. The role of negative control was served by untreated cells. The cellular uptake was quantified by measuring the increase in MFI (mean fluorescence intensity) [26].

**Qualitative through fluorescence microscopy.** Around 0.5 × 10⁶ cells/well were seeded in a 12 well plate and after adherence, the cells were given treatment with C6 alone and C6 loaded nanoparticles having C6 concentration as 10 μg/mL. At various time points of 1, 2, and 4h the wells were washed twice with PBS and observed for qualitative estimation of nanoparticle uptake through C6 tracker dye under the microscope [27].

**Annexin V-FITC assay**

MDA MB 231 (1 × 10⁶) cells were seeded in 6 well plates. After overnight adherence, the cells were then treated with GEM, GSLN, and cGSLN at 30 μM concentration. After 48h of treatment, by addition of trypsin-EDTA cells were harvested and collected after washing with PBS by centrifuging at 5000 rpm. Cells were then treated according to the manufacturer’s protocol with annexin V-FITC and propidium iodide. Apoptosis was detected and analyzed by flow cytometry for the above-treated cells [28].

**Hoechst staining and ROS generation assay**

MDA MB 231 cells with a cell density of 0.5 × 10⁶ cells/well were seeded in a 12-well plate. After the adherence of the cells, they were treated with various formulations at a concentration of GEM equivalent to 30 μM concentration. After 48h of incubation, the changes in nuclear morphology were observed by staining with Hoechst dye (2 μM) and DCFDA (10 μM) incubated at RT for 30min. Finally, the cells were washed watchfully with PBS and examined under microscopy (ZOETM Fluorescent Cell Imager, BIO-RAD) [29,30].

**3D multicellular spheroid inhibition assay**

MDA MB 231 cells (5000 per well) were seeded in an ultra-low attachment 96 well plate. After three days the formed multicellular 3D spheroids were treated with GEM, GSLN, and cGSLN at concentrations of 50 and 100 μM. Spheroids were observed for their morphological changes using microscopy for the next 6 days [31,32].

**Tumor regression study**

In vivo tumor efficacy study was carried out in female BALB/c mice. Animal experiments were performed after attaining the permission from the Institutional Animal Ethics Committee (IAEC) of the CSIR-Indian Institute of Chemical Technology, Hyderabad with the sanction number IICT/26/2018. All studies were conducted according to the stipulated guiding principles of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India). Experimental animals were adapted in the animal facility that is maintained at a temperature and relative humidity of 22 ± 2°C and 50–60%, respectively in a 12:12-h light-dark cycle.

**Induction of tumor**

A syngenic orthotopic breast tumor model was employed using BALB/c mice. Mouse breast cancer cells (4T1) at a cell density of 50,000 cells per 100 μL volume per mice were inoculated subcutaneously (s.c.) into the 4th mammary fat pad [33]. Tumor volume was measured using vernier calipers and calculated according to the formula of (l”b²)/2, where ‘l’ was the major axis and ‘b’ was the minor axis. In parallel, the weights of the mice were also checked twice per week until the end of the study.

**Investigation of tumor targeting potential of GEM loaded nanoparticles by in vivo tumor regression study**

After the development of a tumor and when the volume of the tumor reached 100–150 mm³ mice were divided into four groups containing five mice each. Groups were divided into control, pure drug, GSLN, and cGSLN. Pure drug and formulations were given through an intravenous route at a concentration of 20 mg/kg of the drug twice per week for 2 weeks and then mice were sacrificed after 3 days of last treatment, tumors were isolated, and their weight and volumes were checked [34].

**Statistical analysis**

Most of the experiments were executed in triplicate (n = 3), but in the case of in vivo studies ‘n’ was taken as 5. Graph Pad Prism software (version 5.04) was used for data compilation and analysis. Results were expressed as mean ± standard deviation (SD). Tukey’s multiple comparison test (one way) and Bonferroni’s post-test (two way) ANOVA were used for comparison between groups. (^{*}p < 0.05, ^{*}{*}p < 0.01, ^{*}{*}{*}p < 0.001, and ^{*}{*}{*}{*}p < 0.0001). A value of p > 0.05 represents not significant (ns).

**Results**

**Preparation and physicochemical characterization of solid lipid nanoparticles**

GEM is a hydrophilic drug that can be encapsulated using a double emulsification method employing the sonication energy. The internal aqueous phase (w1) was optimized using three different surfactants pluronic F68, tween 80, and TPGS. It was found that the use of TPGS lead to the formation of nanoparticles with good zeta potential and smallest particle size of 87.1 ± 1.9 nm in comparison to 106.6 ± 2.7 and 107.8 ± 2.5 nm for pluronic F68 and
between 80, respectively. Similarly, two different surfactants, tween 80 and pluronic F68, were used in the external aqueous phase (w2) and found that the use of tween 80 resulted in the formation of the nanoparticles with a smaller particle size of 160.6 ± 2.1 nm and less polydispersity index (PDI) of 0.290 ± 0.02 than those prepared with pluronic F68 which showed 231.6 ± 2.4 nm and 0.338 ± 0.05 for particle size and PDI, respectively. Between the two electrolytes, the use of NaCl (100 mM) resulted in the formation of nanoparticles with good size (76.5 ± 2.2 nm, 0.158 ± 0.02 PDI) in comparison with 100 mM calcium chloride (80.1 ± 1.8 nm, 0.181 ± 0.03 PDI).

In terms of process parameters for primary emulsification, a sonication time of 5 min resulted in the formation of a stable emulsion with less particle size of 78.5 ± 2.1 nm in comparison to 2 min which gave 97.8 ± 2.2 nm sized nanoparticles. Secondary emulsification required 7 min time for the formation of stable and translucent emulsion with a particle size of 75.0 ± 2.6 nm, whereas the sonication times of 3 and 5 min yielded nanoparticles with a particle size of 121.3 ± 3.1 and 98.6 ± 2.3 nm, respectively. The emulsion which was obtained after secondary emulsification was further made up to 10 ml using external aqueous phase and stirred for 3 h for solvent evaporation. From the optimization studies, the final formulation was made up of lipids glyceryl monostearate and stearic acid both comprising 100 mg with lecithin 40 mg as internal emulsifier dissolved in 2 ml of chloroform. The inner aqueous phase was fixed to 2% TPGS and the outer aqueous phase was internal emulsifier dissolved in 2 ml of chloroform. The prepared solid lipid nanoparticles showed monodispersity (PDI < 0.3) and zeta potential was found to be PBS −19.2 ± 1.6, −18.8 ± 1.5, −14.3 ± 1.5 mV for Blank SLN, GSLN, and cGSLN formulations (Table 1). The use of electrolytes NaCl and CaCl2 showed a profound increase in encapsulation efficiency by preventing the drug leakage through maintenance of the osmotic pressure gradient that restricts the movement of the drug across the aqueous phases from inside to outside. Also, the presence of electrolytes in the outer external phase reduces the internal aqueous phase droplet size and thus reduces porosity [35]. Comparatively, NaCl showed more encapsulation (62 ± 2.3%) than CaCl2 (57 ± 1.9%). The % encapsulation efficiencies of the final formulations GSLN and cGSLN were found to be 62 ± 2.3 and 60 ± 1.7, respectively signifying nominal loss of GEM during cRGDFK surface coupling onto cGSLN. TEM analysis showed the spherical morphology of the prepared solid lipid nanoparticles and the size results were in good agreement with the DLS results (Figure 1C).

FTIR analysis shows the signature bands of ureido and hydroxyl groups of GEM at 1689 and 1062 cm⁻¹, respectively. These peaks were prominent in GSLN which indicates the encapsulation of GEM in nanoparticles. In the case of GSLN, the carboxylic acid group was also observed at 1724.28 cm⁻¹ which is then involved in the conjugation reaction with cRGDFK, and peaks at 1568.78 and 1640.33 cm⁻¹ indicate amide bond formation between carboxyl group in SLN and amine group of cRGDFK peptide. In DSC analysis pure GEM showed a melting point at 275 °C corresponding to its melting point. GEM-loaded nanoparticles showed a peak at around 60 °C that is attributed to the melting point of the lipid GMS used in the formulation of the nanoparticles (Figures 1A,B) [36].

### Controlled drug release profile and stability studies of prepared nanoparticles

The drug release pattern of GEM from various dialysis bags containing pure GEM and GEM-loaded formulations in different buffers was evaluated in vitro. GEM release was more than 90% within 6 h for pure GEM in the case of both the media whereas the case of nanoparticles showed initial burst release followed by a controlled release pattern. The initial fast release may be due to the release of GEM present at the surface of the nanoparticles or near the periphery of nanoparticles. The succeeding release of the drug may be attributed to the dissemination of the drug through the SLN matrix, or due to lipid digestion, or a combination of both. At the end of 24 h, nearly 43 ± 1.5% and 40 ± 2.0% of the drug were released from the GSLN and cGSLN, respectively in PBS buffer while in SAB the release of GEM was 51 ± 4.7 and 50 ± 2.6% for GSLN and cGSLN, respectively (Figure 2).

The prepared nanoparticle formulations were evaluated for their colloidal and steric stabilities. Colloidal stability was assessed by checking the change in particle size and zeta potential in various media, such as normal saline (NS), PBS, serum, and distilled water at various time points. Results in Figures 3(A,B) show that there is minimal change in the particle size after dispersion in serum which is very important for the formulations which are intended for administration through an intravenous route. In addition, the change in particle size was observed in the case of NS and PBS but in the case of distilled water, the change was less when compared to NS and PBS. The order of change in zeta potential was found to be PBS < NS < Plasma < distilled water.

The steric stability of the prepared nanoparticles was determined with the use of a flocculating agent, i.e. sucrose sulfate. It was observed that nanoparticles resisted flocculation from 0.1 to 1.0 M concentration of sucrose sulfate. The planarity of the graph represents the stability of the nanoparticles which is shown in Figure 3(C). Steric stability of the nanoparticles denotes their ability to avoid aggregation which is very important in evading their clearance by macrophages inside the biological system.

### In vitro studies in 2D and 3D cell cultures of MDA MB 231

#### Viability study in MDA MB 231 cells

Anti-proliferation studies were performed in MDA MB 231 cells for different formulations to investigate the cytotoxicity

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**Table 1.** Physicochemical characterization of blank solid lipid nanoparticles (BSLN), GEM loaded nanoparticles (SSLN), and cRGDFK conjugated GSLN (mean ± standard deviation; n = 3).

| Formulation | Particle size (d,nm) | Polydispersity index | Zeta potential (mV) | Drug encapsulation efficiency (%) |
|-------------|----------------------|----------------------|---------------------|----------------------------------|
| BSLN        | 75.03 ± 2.65         | 0.200 ± 0.017        | −19.2 ± 1.62        | –                                |
| GSLN        | 98.57 ± 3.8          | 0.158 ± 0.032        | −18.85 ± 1.48       | 62 ± 2.3                         |
| cGSLN       | 130.1 ± 10.2         | 0.234 ± 0.029        | −14.3 ± 1.53        | 60 ± 1.7                         |
behavior of the pure GEM in comparison with the GSLN and cGSLN. The half-maximal inhibitory concentrations (IC$_{50}$) of all the formulations are displayed in Table 2. Both GSLN and cGSLN showcased IC$_{50}$ values of 32.06 ± 1.85 and 25.50 ± 3.85 µM, respectively in comparison with GEM showing 31.76 ± 3.3 µM (Figure 4).

Figure 1. Physicochemical characterization of GEM, GEM loaded SLN and cRGDFK conjugated GEM loaded SLN using (A) FTIR, (B) DSC thermograms, and (C) TEM image of cGSLN.

Figure 2. In vitro drug release study of GEM, GSLN (GEM loaded SLN), and cRGDFK conjugated GSLN (cGSLN) in (A) SAB pH 5.5 and (B) PBS pH 7.4.
Intracellular tracking of C6 loaded nanoparticles through cellular uptake study

For quantitative estimation of the cellular uptake of the C6-loaded nanoparticles flow cytometry analysis was performed in the MDA MB 231 cell line. Movement of the peak toward the right or change in the fluorescence intensity was observed in cells treated with the solid lipid nanoparticles in comparison to the non-treated cells following a 24 h incubation period. The mean fluorescence intensity of C6 SLN and C6 cSLN were 1,51,428 and 1,75,721, respectively (Figure 5(A)).

In addition, qualitative analysis for cellular uptake of the C6-loaded SLN was also performed to check the time-dependent uptake of the nanoparticles. From the result, it was clear that with the increase in time the cellular uptake of the C6 cSLN was increased and the intensity is more in comparison to the unconjugated SLN which is in good agreement with the quantitative measurement of data obtained with FACs instrument (Figure 5(B)).

Evaluation of anti-cancer activity of nanoparticles in MDA MB 231 cells

Annexin V–FITC/prodipidum iodide dual staining assay was performed to inspect the externalization of phosphatidylserine and to understand whether it is due to programmed physiological cell death or necrosis. In this study, MDA MB 231 cells were treated with GEM, GSLN, and cRGDfK conjugated GEM loaded SLN (cGSLN) for 48 h at 30 μM concentration and examined for the apoptotic effect. GSLN and cGSLN showed higher cellular apoptosis when compared to pure GEM.
Hoechst helps in finding the changes in the nuclear morphology which was usually observed in apoptotic cells. Intense blue color and fragmented nuclei indicate that formulations were bringing about apoptosis and among three formulations, cGSLN showed the highest changes in nuclear morphology indicating that peptide conjugated formulations were more active (Figure 6(a)).

The reactive oxygen species produced in the cells convert the non-fluorescent DCFDA into fluorescent DCF molecule. The intense green color indicates the intensity of ROS in apoptotic cells. The intense green color was observed more in cells treated with cGSLN in comparison to those treated with GEM or GSLN, indicating increased ROS production by ligand grafted SLN because of its more intracellular trafficking through the highly expressed integrin receptors on MDA MB 231 cells. Quantitative estimation was done with ImageJ software where a graph was drawn using the fluorescence signal calculated as CTCF (corrected total cell fluorescence) values (Figures 6(a,b)).

**Multicellular inhibition potential of cGSLN against 3D (spheroids) MDA MB 231 cultures**

3D spheroids provide an excellent model for the tumor in a living organism (in vivo) in comparison with 2D cultured cells (in vitro). Spheroids formation took three days after which they were treated with various formulations at two different concentrations of 50 and 100 μM. At regular time intervals, the treated spheroids were observed for changes in their morphology. It was clearly seen that cGSLN and GSLN were more active in bringing the disassociation of the spheroids in comparison with GEM. Among the GSLN and cGSLN, the ligand conjugated GSLN started to disassociate the spheroids from day 1 and the extent of disassociation increased with the days. In the case of GSLN, disaggregation of spheroids was started on day 3 and continued with the increase in the intensity of disassociation with time (Figure 7).

**Significant in vivo tumor growth suppression mediated by ligand conjugated GSLN**

Tumor regression studies were conducted in BALB/C mice to evaluate the in vivo antitumor efficacies of GEM, GSLN, and cGSLN. As shown in Figure 8(D), tumors quickly grow in the control group to a volume of 1389.0 ± 448.1 mm³ in 28 days of the study. But, cGSLN showed a significant decrease to 168.7 ± 66.9 mm³; p < 0.001 in the tumor volume (Figure 8(A)) compared to GEM (699.0 ± 137.1 mm³; p < 0.01) and GSLN (637.6 ± 184.0 mm³; p < 0.01) that proves the supremacy of targeted drug delivery in the treatment of cancer. This effective decrease in tumor volume can be attributed to the overexpression of integrin receptors that allowed more of the ligand conjugated SLN to carry the anticancer cargo into the cells resulting in more GEM accumulating in the cancer cells and thus showing the efficient anticancer activity as compared to other groups which received the same amount of GEM (pure GEM and GSLN) (Figures 8(A,D)). In the case of weight of the mice the change was not that much significant and the average tumor weights for various groups were found to be 1.10 ± 0.43, 0.60 ± 0.10, 0.53 ± 0.09, and 0.29 ± 0.09 g, respectively for control, GEM, GSLN, and cGSLN groups (Figures 8(B,C)).

**Discussion**

Many serious side effects of GEM make it unsuitable for intravenous injection. In the present study, an attempt has been made to decrease the unwanted side effects by encapsulating it into SLN and then conjugating it with ligands for specific targeting of the.
breast cancer by avoiding off-target side effects. GEM-loaded solid lipid nanoparticles (GSLN and cGSLN) with minimal particle size (<200 nm), narrow PDI, and good zeta potential were prepared. Any formulation having the particle size of below 200 nm greatly favors the passive targeting of the tumor [37]. In addition, the prepared nanoparticles GSLN and cGSLN also avoid the clearance by the mononuclear phagocytic system (MPS) since the smaller-sized nanoparticles are less likely to be cleared by MPS [38]. The decline in the negative zeta potential of cGSLN to $-14.3\, \text{mV}$ could be attributed to a decrease or neutralization in the negative charge of SLN–COO$^-$ group due to surface conjugation with cRGDFK ligand [13]. Both GSLN and cGSLN showed good encapsulation efficiency with the incorporation of electrolyte NaCl in the outer aqueous phase. A slight decrease in the surface charge and FTIR spectra designates the conjugation of cRGDFK onto the solid lipid nanoparticles. In the DSC thermograph, the absence of a peak at 275 $\, ^\circ\text{C}$ in GSLN indicates the phase transition of GEM from crystalline to amorphous which will further aid in its solubility increment. This kind of change in phase transition was also observed in our other nanoformulations [27,32,36].

*In vitro* drug release studies showcased biphasic release patterns. There was a similar trend in drug release profiles of GSLN and cGSLN particles indicating that surface conjugation of SLN with peptide does not impinge on release profiles [13,24,27,36]. It

![Figure 6](image_url)

(a) Hoechst and DCFDA staining on MDA MB 231 cells for GEM, GEM loaded SLN (GSLN) and cRGDFK conjugated GEM loaded SLN (cGSLN) after 48 h of incubation. Scale bar 40 $\mu\text{m}$. (b) Semiquantitative estimation of ROS generation using ImageJ software.
was also observed that drug release from nanoparticles showed sustained release up to 48 h. To check the effect of pH on the release pattern, the formulations were exposed to two different media: SAB pH 5.5 and PBS pH 7.4. Here, the pH offered by SAB represents the pH around the cancer cells. It was observed that the drug release was faster in acidic pH when compared to the

Figure 7. 3D multicellular spheroid inhibition assay for various treatments of pure GEM, GEM loaded SLN (GSLN), cRGDFK conjugated GSLN (cGSLN) at 50 and 100 μM for 6 consecutive days. Images were captured using phase contrast microscopy.

Figure 8. In vivo tumor regression study of orthotopic breast cancer 4T1 cell induced BALB/c mice. (A) Tumor volume growth curve. (B) Change in body weights of the mice. (C) Average tumor weight at the end of the study. (D) Images of isolated tumor tissues showing regression. Data were shown as mean ± SD (n = 5) (*p < 0.05, **p < 0.01, ***p < 0.001).
physiological pH of 7.4. This reason could be attributed to the enhanced therapeutic efficacy of the formulations in the tumor regression study.

A cell viability study performed for BSLN proved its biocompatibility. The anticancer activity of GEM in cGSLN was improved indicating the importance of cRGDfK ligand in enhancing the anticancer potential of GEM through integrin receptors mediated endocytosis [39,40]. C6 cSLN showed increased cellular trafficking in comparison to C6 SLN and C6 alone. This exposes the fact that cRGDfK present on the SLN is helping in more penetration of the nanoparticles through the integrin receptors that are proposed to be overexpressed on the surface of the breast cancer cells (MDA MB 231). The higher cytotoxicity exhibited by cGSLN may be attributed to the truth of higher cellular uptake.

Gemcitabine induces apoptosis by interfering with DNA synthesis. In Annexin V-FITC assay the number of cells in early and late stage apoptosis was more in the case of GSLN and cGSLN when compared to GEM alone. GEM, GSLN, and cGSLN showed 22.6 ± 0.9, 28.4 ± 1.1, and 33.3 ± 0.8% of apoptosis which indicated that cGSLN induced superior cellular apoptosis in comparison with GSLN or pure GEM (Figure 9). This result indicates the fact that the death of MDA MB 231 cells was by programmed cell death but not by necrosis.

Spheroids are aggregates of cells that mimic tumors, they differ from normal 2D cell culture models in having an extracellular matrix, interactions between cell to cell and defined geometry. The results obtained through multicellular inhibition potential of GSLN and cGSLN against 3D spheroids have clearly provided an insight into the potential of cRGDfK conjugated GEM loaded SLN in targeting and disrupting the tight 3D MDA MB 231 spheroids. The in vivo tumor regression study exhibited the significant decrease in the tumor volume by cGSLN which is in good agreement with the multicellular 3D spheroid inhibition activity displayed by cGSLN that can be accounted to the cRGDfK ligand that helped in taking the payload into the tumor [41,42].

Conclusion

The successful synthesis of the $\alpha_v\beta_3$ receptor targeting SLN (cGSLN) was evident from the physicochemical characterization techniques performed in the present study. The formulated nanoparticles were evaluated for their in vitro anticancer efficacy through 2D and 3D cell culture studies and were found to be more efficacious when compared with pure GEM. The in vivo tumor regression studies, performed in an orthotopic breast
cancer model in BALB/c mice, proved clearly the significance of the targeted delivery systems in the delivery of anticancer drugs to the tumor of interest.

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**Author contributions**

The manuscript was written with the contributions of all authors. All authors have approved the final version of the manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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