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Research Article

In Vitro Studies of Prednisolone Loaded PLGA Nanoparticles-Surface Functionalized With Folic Acid on Glioma and Macrophage Cell Lines

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Abstract

Background: Glucocorticoids are employed for their anti-inflammatory effects in treating glioma, whose cells are known to overexpress the folate receptors. Some glucocorticoids have shown inhibitory effects, but the efficacy of prednisolone when delivered *via* folate receptor-mediated uptake, has not been attempted. The study aimed to assess the efficacy of targeted delivery of prednisolone on glioma cell lines like C6 and U87 *via* the folate receptors.

Methods: Targeted delivery of prednisolone was achieved by initially conjugating folic acid (FA) to the di-block copolymer of polylactic acid (PLA) – polyethylene glycol (PEG). This moiety carrying di-block copolymer was incorporated on the surface of the drug-loaded poly lactic-co-glycolic acid (PLGA) nanoparticle (NP) by employing the Interfacial Activity Assisted Surface Functionalization (IAASF) technique. The NPs were evaluated for size, zeta potential, and drug loading. It was characterized using particle size analyser, SEM, ^{1}H-NMR, and XRD. cell uptake, cytotoxicity, and anti-inflammatory activities were studied for various formulations.

Results: The cytotoxicity assay indicated a high cell growth inhibitory effect of drug encapsulated NPs with FA moiety as compared to free drug and NPs without the moiety for an incubation period of three, five, and six days. The growth-inhibitory effect of the free drug was short-lived, whereas FA functionalized NPs showed higher uptake and sustained inhibitory effect, and were also able to significantly control the release of pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF-α) and nitric oxide (NO).

Conclusion: Uptake, attenuation of pro-inflammatory signals, and the inhibitory effect of prednisolone on the cells were more effective when targeted with the FA moiety on the surface of NPs as compared to free drug and NPs without the moiety.

Introduction

Glioma is a term employed to categorize tumours of the central nervous system (CNS) that owe their origin to the glial cells.[1] Many of these tumours exhibit high rates of infiltration and growth and are known to be life-threatening.[2] Treating them with combination therapy is also not very beneficial.[3,4] Enhanced permeability and retention (EPR) effect in tumour tissue is known to assist the accumulation of the drug-loaded carriers,[5,6] but entering the cell with ease poses a challenge.[7] Recent studies have shown that receptors of folate get overexpressed on cancer cells[8,9] and activated macrophages.[10] Employing these receptors as a target to deliver the drug is a promising area where many have ventured.[11-14] Thus, the delivery of drugs, encapsulated in a biodegradable polymer through receptor-mediated endocytosis could help in inhibiting the cell proliferation.

Glucocorticoids (GC) have been the preferred class of drugs for numerous inflammatory-related and autoimmune diseases.[15,16] Their contribution to treating gliomas have been limited to their edema reducing abilities. However, recent studies[17] have highlighted their effectiveness in treating acute lymphoblastic leukemia[18] or CNS lymphoma.[19] Prednisolone is a popular GC that is known to be less potent, and its usage is known to be accompanied by a lesser degree of side effects like depression, mood swings, and insomnia.[20,21] Some GC's have shown to enhance necrotic cell death induced by serum deprivation[22] due to the activation of glucocorticoid receptors (GR), and the resultant GC-GR complex triggers different signaling pathways, resulting in variable outcomes.[22-26] Studies evaluating the effect and efficacy of prednisolone on glioma have been very few,[27,28] and targeted delivery of prednisolone encapsulated in PLGA NPs have not been studied.

PLGA is a well-known FDA approved carrier for drug delivery but lacks active functional groups.[29] PLA-

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based NPs have shown slightly higher drug-loading of hydrophobic drugs as compared to PLGA NPs, but the slight hydrophilic nature of PLGA NPs contribute to a higher cumulative percentage release of the encapsulated drug.[30,31] PEGylation of NPs not only improves the stealth abilities but also provides active functional sites for ligand conjugation that assist in the targeted delivery of drugs.[29] In PLA-PEG based NPs, the PLA content determines the size and drug loading, whereas the PEG content determines the release profile.[32] Longer PEG chains lead to more stable NPs. However, an increase in the PLA chain length improves the drug loading, but deteriorates NP stability and sometimes causes condensation in solvents.[32] Higher drug-loading of hydrophobic drugs in PLA-PEG NPs results in faster release of drug and also causes instability in the PLA-PEG structure.[32] This instability of PLA-PEG NPs leads to lower uptake in cells when compared to PLGA NPs.[33] In our previous work,[34] we demonstrated high drug loading and a sustained release of prednisolone via PLGA NPs. Patil *et al.*[29] demonstrated a very simple method known as Interfacial Activity Assisted Surface Functionalization technique (IAASF),[29,35] to incorporate different targeting molecules on the surface of PLGA NPs. The process of functionalization was based on the principle of self-assembly, where the hydrophobic part (PLA) of the di-block copolymer of PLA-PEG would stay in the PLGA NP, whereas the hydrophilic PEG chain along with the targeting moiety would remain on the outer surface of the NP during the solvent evaporation step. This simple method was employed to surface-functionalize FA on the prednisolone-encapsulated PLGA NPs. The di-block copolymers of PLA-PEG was initially synthesized and then conjugated with FA (PLA-PEG-FA), which was then incorporated on the PLGA NPs. These NPs were characterized for size, zeta potential, and drug loading, followed by uptake studies to analyse the specificity of receptor-assisted uptake. Cytotoxicity study was carried out on different glioma cell lines and cytokine (TNF-α), and NO release was analysed to test the efficacy of the NPs with and without the FA moiety on the surface when compared to free drug.

Materials and Methods

Poly (D, L-lactide-co-glycolic acid (50:50) (MW: 100000-120000) was procured through Durect Corp. AL, USA. Polyethylene glycol (PEG; MW-4000), monomethyl ether PEG (mPEG; MW-5000), polyvinyl alcohol (PVA; MW: 13000), α-Amine-ω-hydroxy PEG (Mw-3500), N-hydroxysuccinimide (NHS), L- lactide, folic acid, coumarin-6, 4′,6-diamidino-2-phenylindole (DAPI), lipopolysaccharide (LPS), 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), cellulose acetate membrane (MWCO-1000 Da) and prednisolone were purchased from SIAL (MERK, India). Benzoic acid, methanol, dicyclohexylcarbodimide (DCC), triethylamine (TEA), sodium chloride (NaCl), tri-fluoro acetic acid (TFA), dimethyl sulfoxide (DMSO), dichloromethane (DCM), acetonitrile, and diethyl ether were purchased from Loba Chemie, Mumbai. Regenerated cellulose acetate dialysis tubing (MWCO-6000 to 8000 Da) was procured from Orange Scientific, Belgium.

Ham's F-12K, DMEM medium, fetal bovine serum (FBS), trypsin-EDTA, Triton-X 100, penicillin-streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and NO assay kit were purchased from HiMedia (Mumbai). The TNF-α (Rat) ELISA kit was procured from Krishgen BioSystems (Mumbai). Rest of the chemicals and reagents were of analytical grade and purchased from reputed sellers.

Synthesis of PLA-PEGm

mPEG-5000 (0.2 g) was used as a macroinitiator for the ring-opening polymerization of cyclic ester lactide ((3S)-Cis-3,6-Dimethyl-1,4-dioxane-2,5-dione) (0.8 g).[36] The reactants were dissolved in 20 ml of dry methylene chloride, and 20 µl of DBU was added to the solution and stirred under nitrogen at room temperature (RT) for two hours. Benzoic acid (10 mg) was added to this reaction mixture and stirred for ten more minutes to arrest further polymerization. The mixture was reduced to around 5 ml using a rota-evaporator and precipitated into a beaker containing 200 ml of ice-cold ether. The precipitate was dried and filtered to get Di-block copolymers of PLA-PEGm.

Synthesis of NHS-FA

The preparation of NHS-FA was carried out as described by Patil *et al.*[29] Briefly, NHS-FA was prepared by adding folic acid (0.25 g) into a mixture of anhydrous DMSO (10 ml) and TEA (0.2 ml). This solution was stirred under anhydrous conditions in dark for 18 hours. DCC (0.12 g) and NHS (0.067 g) were added to this mixture and further stirred in dark for 18 hours. The precipitate was filtered out and the solvent evaporated under vacuum. The product obtained (NHS-FA) was employed in the formulation of PLA-PEG-FA copolymer.

Synthesis of PLA-PEG-FA

α-Amine-ω-hydroxy PEG (0.2 g) was used as a macroinitiator for the ring-opening polymerization of cyclic ester lactide (0.8 g) in the presence of DBU as mentioned earlier, to obtain PLA-PEG-NH_2. Further, PLA-PEG-NH_2 (0.6 g) and TEA (20 µl) in DMSO (10 ml) was stirred overnight, and later NHS-folic acid was added to this mixture. The reaction mixture was stirred in the dark under N_2 atmosphere for another 20 hours.[29] The unconjugated folic acid molecules were removed by dialysis (MWCO 1000) and the dialyzed product was lyophilized and analysed for folic acid conjugation by ^{1}H-NMR spectroscopy. The scheme of synthesis is provided in Figure 1.

Synthesis of surface-functionalized PLGA NPs by IAASF technique

PLGA 50:50 (0.05 g) and prednisolone (0.025 g) were

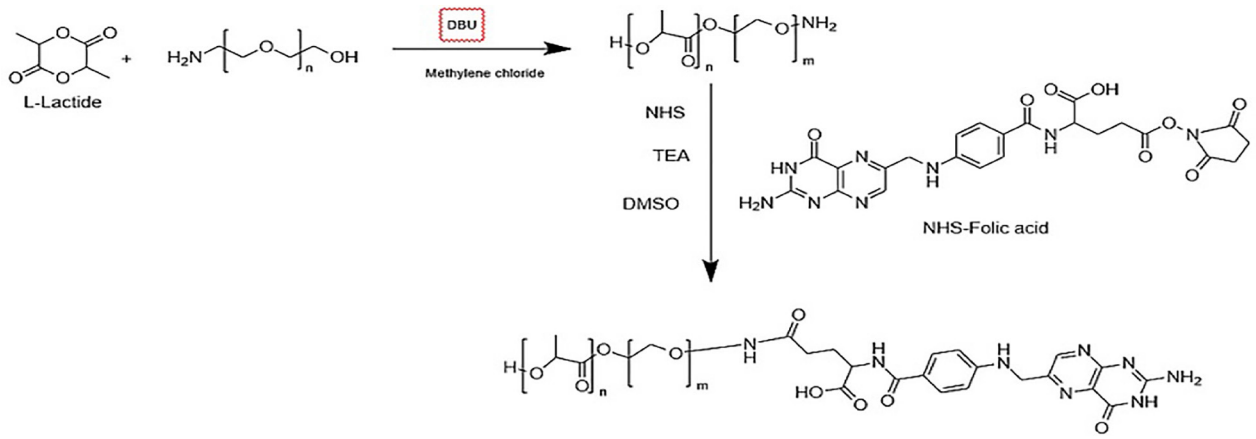

Figure 1. Scheme of synthesis of PLA-PEG-FA copolymer.

dissolved in 1.5 ml of chloroform. An oil-in-water emulsion was formed by emulsifying the polymer solution in 15 ml of 2.5 % w/v aqueous PVA solution using a probe sonicator for eight minutes over an ice bath. The di-block copolymers of PLA–PEG conjugated to folic acid were dissolved in chloroform (10 mg in 100 µl) and added to the above emulsion with stirring. The resultant emulsion was then further stirred for 18 hours at ambient conditions to remove the chloroform.[29] The NPs were recovered by centrifugation (15000 rpm) and washed three times with deionized water to remove excess PVA. This was followed by lyophilization, prior to storage. A similar process was employed to obtain Coumarin-6 loaded PLGA based NPs by replacing the drug with coumarin-6, a fluorescent molecule. Henceforth, the folic acid-surface functionalized PLGA NPs (PLGA-PLA-PEG-FA) will be addressed as FA NPs, and PLGA NPs incorporated with di-block copolymer PLA-PEGm (PLGA-PLA-PEGm) will be addressed as PLGA-PEGm NPs.

Characterization of the nanoparticles

The formulated NPs were characterized for size and zeta potential on a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, UK). The integration of the moiety to the PLGA-based NPs was determined by ^{1}H-NMR (Bruker AV-400). The morphology of the NPs was assessed through a scanning electron microscope (SEM).

Drug loading

The drug-loaded NPs (1 mg) were dissolved in 1 mL methanol (triplicate) and kept on a rocker shaker for 48 hours. After centrifuging at 10000 rpm for 20 minutes, the supernatants were kept overnight for methanol evaporation. These samples were dissolved in 1 mL acetonitrile and analysed using RP-HPLC (SPD-20A, LC-20AD-SHIMADZU). The amount of drug loading was determined by using equation 1.

$$Drug\ loading = \frac{\text{Mass of drug}}{\text{Mass of NPs}} \quad \text{Eq. (1)}$$

Cell culture

C6 (rat) and U87 (human) are popular glioma cell lines, with many researchers employing them to study the anti-tumour effects of their formulations.[2,22,24,37] We wanted to check the efficacy of our new formulation on these glioma cell lines. RAW 264.7 is a well-known macrophage cell line. Macrophages are known to over-express the folate receptors when activated.[10,38] RAW 264.7 macrophage cell line was used to check the folate receptor-mediated uptake efficiency of our NPs. For this, C6, U87, and RAW 264.7 cell lines were obtained from NCCS, Pune, India. C6 cells were grown in T25 flasks using Ham's F-12K media with 10% FBS and 1% penicillin-streptomycin, in an incubator at 37°C and 5% CO_2. Similarly, U87 and RAW 264.7 were grown using DMEM as the medium. The media was changed every two to three days and the cells were split when 70 to 80% confluent, using 0.25% trypsin-EDTA.

Uptake studies

Qualitative analysis of uptake

To visualize the particle uptake, C6 cells were seeded and allowed to attach for 24 hours. The media was removed and washed two times with phosphate-buffered saline (PBS). These cells were then incubated with different coumarin-6 loaded NPs (coumarin-6 concentration of 1 µg/ml) for 30 minutes and washed three times, followed by DAPI (500 ng/ml) staining for ten minutes. The cells were then observed under an Olympus IX73P1F fluorescence microscope.

Quantitative analysis of uptake

Studies were carried out on C6, U87, and RAW 264.7 cell lines to quantify the uptake in terms of the mass of Coumarin-6 per mass of cell protein. Briefly, about 50000 cells were seeded in each well (24 well) and allowed to attach overnight. A working volume of 0.5 ml/well was maintained. Different treatments were prepared with Coumarin-6 encapsulated in PLGA, PLGA-PEGm, and FA NPs and characterized. A concentration of 1 µg/ml of Coumarin-6, encapsulated in NPs was maintained for all treatments. Control wells received equivalent amounts of free Coumarin-6. The media was removed and incubated

with the treatments for 30 and 120 min. This was followed by a gentle PBS wash (twice) and the cells were trypsinized, aspirated and collected in vials. The trypsin was removed after centrifugation. Lysis buffer (400 µl) was added to each sample, vortexed intermittently five times under ice-cold conditions. The samples were then centrifuged (10000 rpm, 4 °C) for 30 min and 100 µl of the sample was kept aside for protein content analysis using Bradford's reagent. To the rest, 700 µl of methanol was added to extract the fluorescent molecule. The vials were kept in dark on a shaker overnight and analysed for the Coumarin-6 content using a microplate reader with a fluorescent detector kept at 458 nm (Excitation) and 512 nm (Emission). Bradford test was optimized by adding 200 µl of Bradford reagent to 10 µl of the sample. The samples were incubated in the dark for ten minutes at room temperature and read at 595 nm.

Cytokine release studies

Cell treatment

C6 glial cells were seeded in 24 well plates with a cell density of 50000 cells/well. A working volume of 0.5 mL/well was maintained throughout the experiment (n = 4). After 24 hours of incubation, the media was aspirated from all the wells and washed with PBS. This was followed by the addition of 0.5 ml of serum-free media (SFM) containing 1 µg/ml of LPS (SFM+LPS) to activate the glial cells and macrophages to produce cytokines. The negative control wells received only SFM. After incubating for 30 min, the media was aspirated and replaced with SFM+LPS, containing 5, 10, and 15 µg/ml of free drug (prednisolone) and FA NPs with equivalent drug concentrations. The control wells received no drug treatment. After two hours of further incubation, the media was aspirated and gently washed with PBS. The wells were refilled with SFM+LPS and incubated further for a period of 24, 48, and 72 hours. The culture media was collected at these time intervals, centrifuged at 1400 rpm for five minutes and the supernatant was stored at -80° C for further analysis.

TNF-α ELISA

TNF-α cytokine production by C6 glial cells was analysed using an ELISA kit according to the manufacturer's instructions and as mentioned previously.[34,39] Briefly, 100 µl of the samples were added to designated wells and incubated at room temperature for two hours. The solutions were thoroughly aspirated and washed four times with wash buffer. Later 100 µl of Rat TNF-α Biotin conjugate solution was added to all wells except the blanks and further incubated at room temperature for one hour. The solutions were again thoroughly aspirated and washed four times with the wash buffer. Next, 100 µl of streptavidin HRP was added to the wells, incubated at room temperature for 30 minutes, and later washed four times with the wash buffer. Finally, 100 µl of stabilized chromogen was added and incubated for 30 more minutes in the dark, followed by the addition of an equal quantity of stop solution, turning the blue solution to yellow. The absorbance was read immediately at 450 nm and the unknown concentrations were determined from the standard-fit curve.

NO release assay

Nitrite production was measured by using the EZAssay Nitric Oxide estimation kit according to the manufacturer's instructions.[34] In brief, 0.1 mL of supernatant was mixed with 0.05 mL Griess reagent I and then with 0.05 mL of Griess reagent II. The absorbance was measured at 580 nm and 630 nm and the concentration was determined from the calibration curve obtained from the nitrite standards.

Cell cytotoxicity assay

C6 and U87 cells were seeded in 96 well plates with a cell density of 5000 cells/well and a working volume of 0.1 ml. The short and long-term effects of the formulations were analysed using the MTT assay. Short-term effects were analysed by removing the treatment after a predetermined time interval (30, 120, and 240 min.) and replacing the wells with media after a gentle wash. Whereas for long-term exposure, the treatment was present for all the days until the cytotoxicity analysis was performed. Briefly, media was removed from all wells and 100 µL of MTT was added to each well and incubated for a period of three hours. The MTT solution was replaced with 100 µL of DMSO to dissolve the formazan crystals and the absorbance was recorded using a Thermo Scientific Varioskan lux microplate reader at 570 nm. The cell viability was assessed in terms of the percentage of positive control.

Statistical analysis

All data, unless specified, are presented as mean ± SD and analysed using t-test or ANOVA in Graph Pad Prism statistical analysis software (Version 7.0), with *p*-value less than 0.05 considered as statistically significant.

Results and Discussion

Characterization of PLA-PEGm

Di-block copolymers of PLA-PEGm were synthesized and characterized using ^{1}H-NMR (Additional file 1 in supplementary data). The molecular weight was calculated by employing the integrated values of the NMR resonance peaks at 3.57 ppm for PEG and 5.2 ppm (CH) or 1.5 ppm (CH_3) for PLA.[40] The values were in close agreement (1.5 or 5.2 ppm) and this was further supported by GPC (Additional file 2 in supplementary data), with less than 5% deviation (Table 1). Crystallinity was assessed using XRD (Additional file 3 in supplementary data), with pure PEG as a standard. A decrease in peak intensity was clearly visible in the block copolymers with an increase in the PLA content. Pure PEG showed prominent diffraction peaks at 2θ = 14, 19, 23, and 27° whereas PLA showed a broad distributed signal from 10 to 24° clearly indicating its amorphous nature. Di-block copolymers with lower PEG content exhibited similar peaks in a subdued manner.[40]

Table 1. Molecular weights determined from ^{1}H-NMR and GPC.

Sample	Di block copolymer	M. Wt. of PLA (approx.)	M. Wt. of polymer (approx.)
1	$mPEG_{5000}$:PLA_{5826}	5826	10826 (10538)*
2	$mPEG_{5000}$:PLA_{8477}	8477	13477 (13531)*

*(M. Wt.) – Determined through GPC.

Characterization PLA-PEG-FA

The FA functionalized di-block copolymer was characterized using ^{1}H-NMR (Figure 2). The spectrum indicated the presence of PEG (OCH_2CH_2: 3.5 ppm), PLA (CH_3: 1.48 ppm and CH: 5.2 ppm), and FA (pteridine ring of FA: 8.648, para-amino benzoic acid group protons at 7.6, 6.6 ppm), validating the attachment of FA to the di-block copolymer.

Characterization of surface-functionalized PLGA NPs

FA NPs were synthesized by incorporating the FA conjugated di-block copolymers, prepared from heterobifunctional PEG on the PLGA NPs using the IAASF technique. The characterization of the NPs was done using ^{1}H-NMR. The spectrum for drug-loaded FA NP (Additional file 4 in supplementary data) indicated the presence of PLGA polymer (CH_3: 1.56 ppm, CH_2: 4.9 ppm, CH: 5.2 ppm), prednisolone (CH_3: 1.47 ppm, CH_2: 2.3 ppm, CH: 5.9, 6.2, 7.3 ppm), and FA (pteridine ring of FA: 8.648, para-amino benzoic acid group protons at 7.6, 6.6 ppm). The spectrum for drug-loaded PLGA-PEGm NP (Additional file 5 in supplementary data) also showed all the peaks of FA NP, except for the peaks of FA.

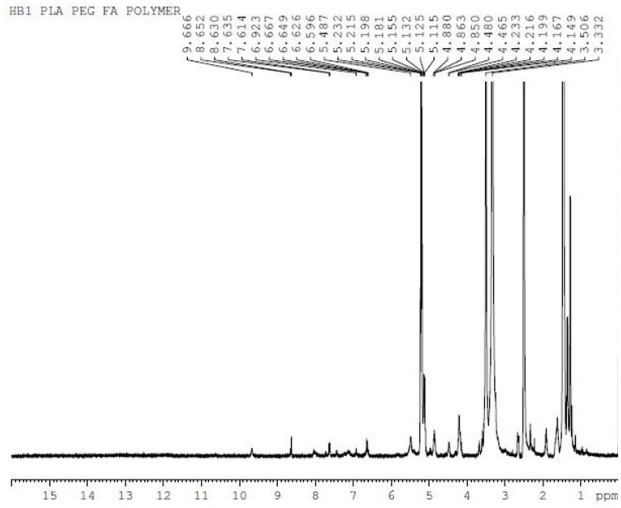

Figure 2. ^{1}H-NMR spectrum of FA functionalized PLA-PEG di-block copolymer.

Characterization of the nanoparticles

The amount of drug loaded in NPs was determined using the same method as previously reported.[34] The formulated NPs with and without the FA moiety on the surface were characterized for size, zeta potential (Figure 3), and drug loading (Figure 4). The FA NPs and PLGA-PEGm NPs were found to have an average size of 326.1 and 358.7 nm, zeta potential of -11.9 and -10.3 mV, and an average drug loading of 104 and 86 µg/mg of NP respectively. SEM analysis of the NPs revealed that the morphology of the particles was fairly spherical (Additional file 6 in supplementary data). There was a slight increase in the size of these surface-modified NPs as compared to plain PLGA NPs.[34] The increase in size could be attributed to the addition of the FA functionalized block copolymers of PLA-PEG on the surface of the NPs. A slight decrease in drug loading and zeta potential was observed after the surface functionalization process. PEGylation of the NPs could have led to a decrease in the Zeta potential of the NPs.[41]

Uptake studies

The uptake of NPs was tracked using coumarin-6 (fluorescent molecule) loaded NPs using a fluorescence microscope. The FA NPs were taken up extensively by the cells (Figure 5) and appeared to be surrounding the nucleus in larger numbers. Quantitative uptake studies were conducted on glioma cell lines like C6 (Figure 6A), U87 (Figure 6B), and macrophage cell line RAW 264.7 (Figure 6C), since activated macrophages[38] and many glioma cells[42] are known to over-express the folate receptors. The uptake of FA NPs was significantly ($p<0.01$) higher when compared to all other NPs (six-fold higher compared to PLGA NP, three-fold higher compared to PLGA-PEGm NP). An increase in the fluorescent molecule uptake was

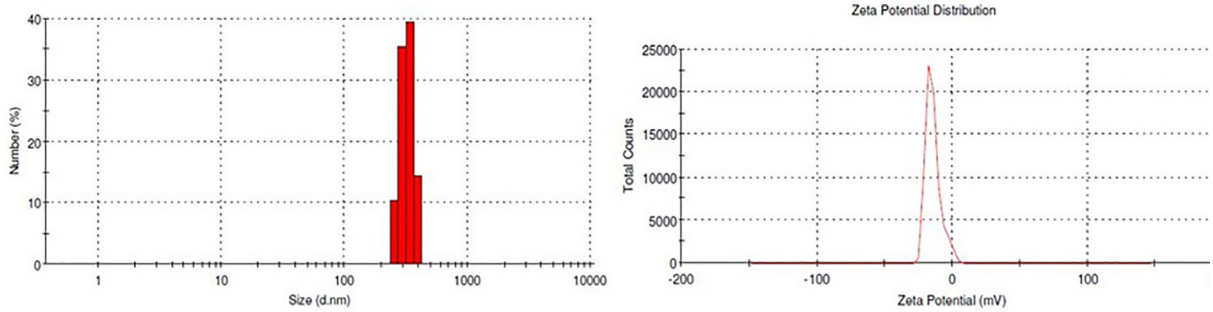

Figure 3. Size distribution and zeta potential of the FA NP.

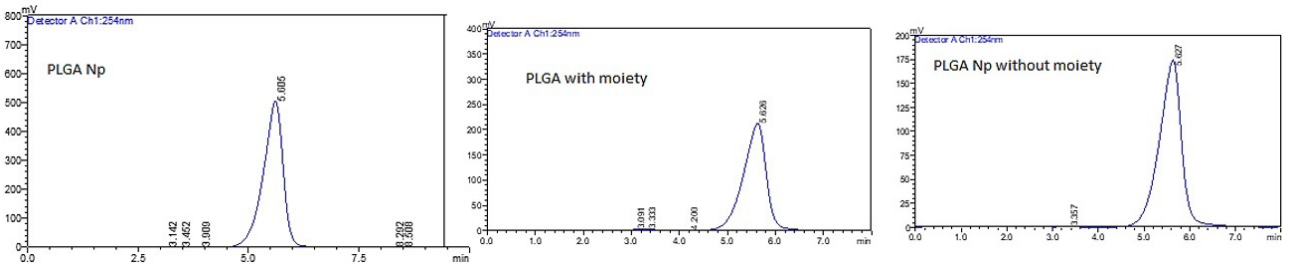

Figure 4. RP-HPLC peaks of drug encapsulation in different NPs (PLGA, PLGA-PLA-PEGm and PLGA-PLA-PEG-FA NPs).

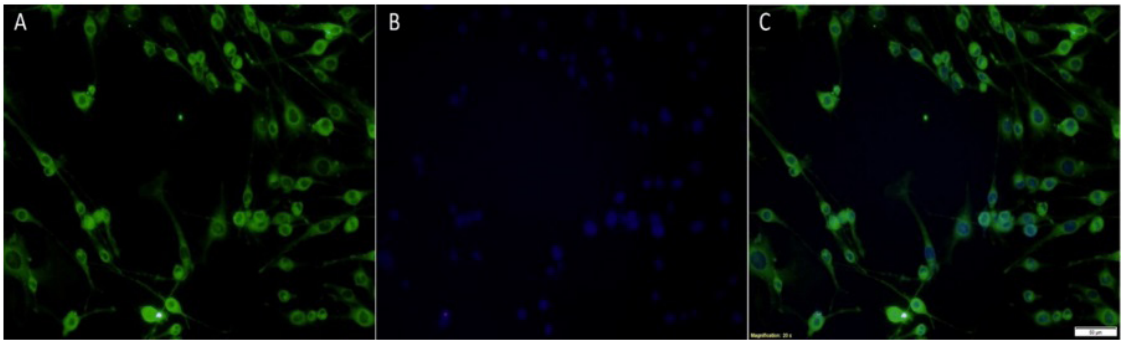

Figure 5. Uptake of Coumarin-6 loaded NPs in C6 cells (A), DAPI (B) and Merged (C).

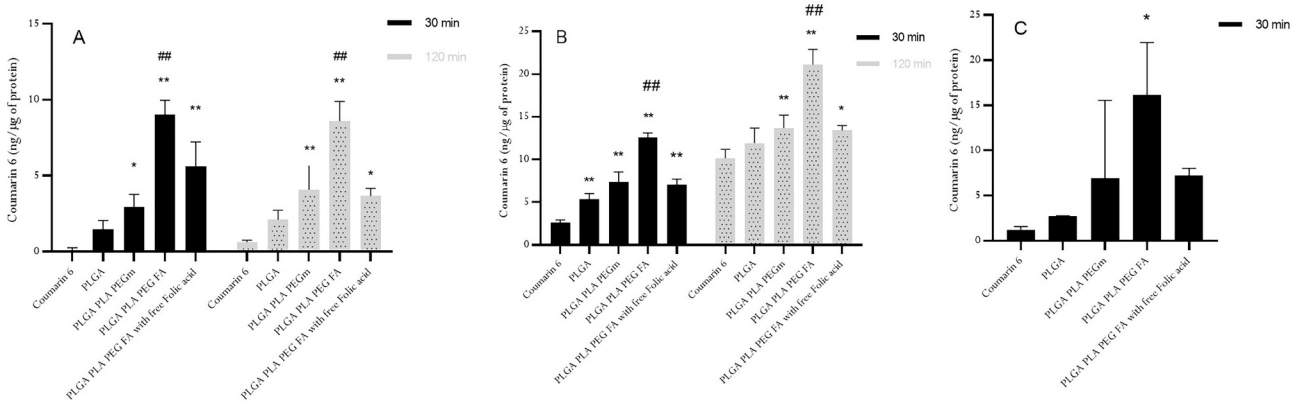

Figure 6. Uptake of Coumarin-6 in C6 cells (A), U87 (B) and RAW 264.7 (C). Values are in mean ± SD (n = 3) with * ($p < 0.05$), ** ($p<0.01$) vs control group (Coumarin-6), # ($p < 0.05$), ## ($p<0.01$) vs group without FA moiety (PLGA PLA PEGm).

seen with higher incubation time. Similar results were also observed on U87 cell lines (Figure 6B) with FA NPs showing significantly ($p<0.01$) higher uptake in comparison to PLGA NPs (2.4 times) and PLGA-PEGm NPs (1.7 times). Uptake studies for RAW 264.7 macrophage cell line (Figure 6C), was done for a 30 minute incubation time. As noticed in glioma cells, FA NPs uptake was significantly ($p<0.05$) higher than free coumarin-6 (13.1 times) and considerably high but not statistically significant, when compared to PLGA NPs (5.9 times) and PLGA-PEGm NPs (2.3 times). To support the fact that FA NPs were predominantly taken in through the folate receptor-mediated endocytosis, excess free-folic acid (1 µM) was employed along with the FA NPs to block the folate receptors. The results showed a significant ($p<0.05$) reduction in uptake of the FA NPs in the presence of free FA. The uptake of PLGA-PEGm NPs was higher than PLGA NPs, but not statistically significant.[43,44] The results predominantly supported the general hypothesis that overexpressed folate receptors on these cells assist in a higher uptake of particles through the receptor-mediated endocytosis process and thereby targeted drug delivery would be a preferred method of delivering the drug to the affected site.

Cytokine release studies

Tumour cells are known to overexpress pro-inflammatory signals like NO and TNF-α. This results in a higher interaction of tumour cells with macrophages and other non-tumour cells, causing tumour cells to proliferate further.[45] A control over the release of these signals will invariantly help reduce further growth of the tumour cells. The amount of TNF-α released (Figure 7) due to LPS-induced cytokine production was measured using an ELISA (Rat) kit. To validate the efficacy of biopolymer-based targeted delivery as opposed to free drug, the effect of prednisolone at different concentrations (P 5, 10, 15 µg/

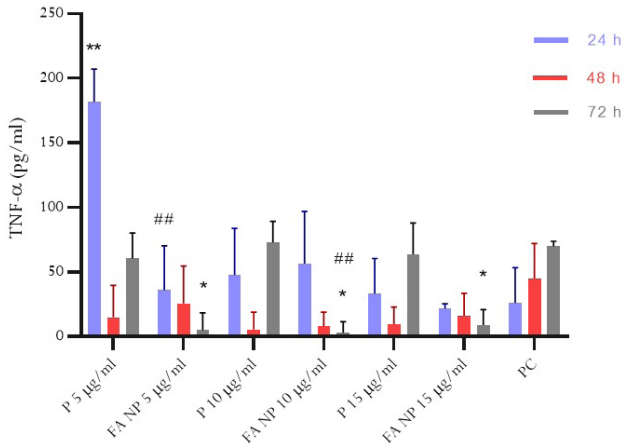

Figure 7. Effect of prednisolone and FA NPs on LPS induced TNF-α production in C6 glial cells. Values are mean ± SD, n=3, * (p < 0.05), ** (p<0.01) vs group of LPS; # (p < 0.05), ## (p<0.01) vs group of Prednisolone in same concentration.

ml) and an equivalent amount of drug-loaded FA NPs (FA NP 5, 10, 15 µg/ml) on the cytokine release was studied.

A decrease in cytokine levels was seen in free-drug treated groups on day two (48 hours) but this was followed by a marked resurgence on day three (72 hours). At the same time, FA NPs showed an incremental decrease in the TNF-α release with each passing day. FA NPs did show a time-dependent effect, but a concentration-dependent effect could not be conclusively established. GC induced necrosis and the absence of serum could have augmented the LPS-induced cytokine production.[22] The decrease in the effects of prednisolone on curtailing the release of TNF-α on latter days (72 hours) could be due to its short half-life.[27] Control groups (PC) showed a steady increase in the TNF-α release on all three days.

NO release (Figure 8) from all prednisolone-treated groups showed a steady increase in levels with an increase in incubation time, whereas FA NP treated groups showed significantly (p<0.01) lower levels.[46-48]

Free drug-treated groups almost mimicked the positive control group, showing a minimal inhibitory effect on

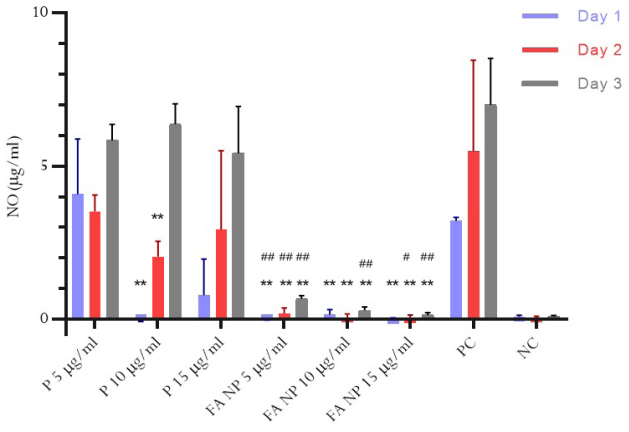

Figure 8. Effect of prednisolone and FA NPs on LPS induced NO production in C6 glial cells. Values are mean ± SD, n=3, * (p < 0.05), ** (p<0.01) vs group of LPS; # (p < 0.05), ## (p<0.01) vs group of Prednisolone in same concentration.

the release of the pro-inflammatory signals. As reported previously,[49-52] the expressed NO levels are low in C6 cells due to possible attenuation of iNOS expression when induced by LPS alone. Overall, a better attenuation of pro-inflammatory signals was observed with FA NP treated groups when compared to groups treated with free drug.

Cell cytotoxicity assay

To demonstrate the variable inhibitory effects of different drug-loaded NPs when compared to free drug, we conducted cytotoxicity studies in serum-containing media for six days on C6 and U87 cell lines. Cell viability was noted on days one, three, five, and six. The long-term effect of the drug and equivalent drug encapsulated FA NPs on C6 cells (Figure 9) was initially carried out by keeping the drug and FA NPs in the medium throughout the course of the experiment. It was observed that the free drug had a relatively better inhibitory effect (P15 - 39.5%) than the FA NPs (FA NP15 - 19.1%) on the initial day (day 1). This inhibitory effect of the free drug appeared to max out on day 3 (71.5%), and for FA NPs on day 6 (71%). However, this effect of the drug encapsulated FA NPs was more sustained and improved as time progressed. The slow and sustained release of the drug from the NPs could have led to a lower inhibition on the initial days when compared to free drug. In the case of free drug, the inhibitory effects were limited to day one and three, whereas a recovery in viability was observed on the latter days (day 5, 6).[28,53,54] The presence of GC receptors[22] on C6 cells may have led to a better uptake of the free drug, thereby limiting the advantage of folate receptor-mediated endocytosis.

The cytotoxic effect of the free drug on U87 cell line (Figure 10) was markedly different, as the effect of the

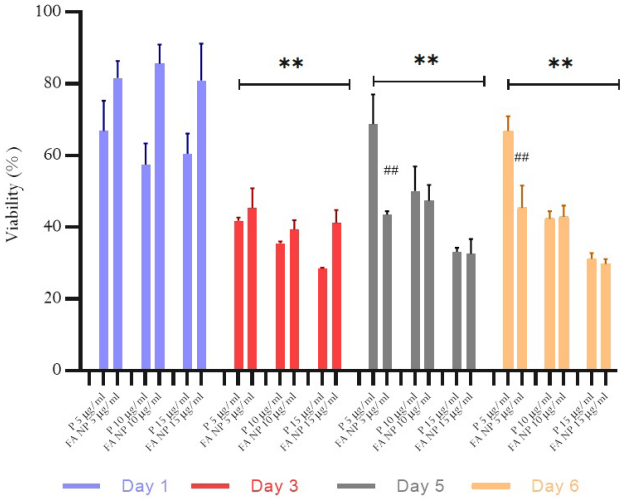

Figure 9. Effect of prednisolone and FA functionalized NPs on cell viability in C6 glial cells. Specific groups were treated with free drug prednisolone (P 5, 10, 15 µg/ml) and the FA NPs with equivalent drug concentrations (FA NPs 5, 10, 15 µg/ml) for different time periods (Day 1, 3, 5, 6). The cell viability was determined as the percentage of control. Values are mean ± SD, (n=5) with * (p < 0.05), ** (p<0.01) vs control group; # (p < 0.05), ## (p<0.01) vs group of prednisolone in same concentration.

free drug was almost negligible initially (day 1), and was significantly lower ($p<0.05$) when compared to the FA NPs on all the days. The inhibition was higher for a higher concentration of the free drug (P10 and P15 ~50 %), with a slight recovery being observed on day 5 and 6, but groups treated with FA NPs maintained high cell inhibition rate (40 to 91.9%) throughout the test period. Time and concentration dependent effects were evident in the FA NP treated groups. U87 (human) and C6 (rat) are well-known glioma cell lines of CNS, both containing the wild type p53 gene.[55,37] The FA NPs showed a similar inhibitory effect on both, but the effect of the free drug was minimal on U87. This may be due to the activation of the GR's by the GC's and its multifaceted effect on cell survival.[26,56] Some GC's have shown to inhibit cell growth[23] in cells and also similar effects were observed in the cells that retained the wild type p53 protein.[57,58] Similarly, studies[59] have shown that GR's activated by GC, play a role in protecting the cells by promoting the sequestration of p53. Further studies will be required to conclusively identify the multifaceted effects of prednisolone on these cell lines.

To assess the efficiency of FA NPs when compared to free drug and PLGA-PEGm (moiety free) NPs, the cells (C6) were tested for the cytotoxic effect when exposed to short term treatment (30, 120, and 240 min). Very short exposure (30 minutes) to treatment showed a relatively good level of inhibition (Figure 11A) and this effect was not only maintained throughout the latter days by FA NPs, but was significantly ($p<0.05$) better than the PLGA-PEGm NPs on day 5 and 6. Overall enhanced effectiveness could be attributed to higher uptake of FA NPs through receptor-mediated endocytosis when compared to the diffusion-based process for the free drug and endocytosis for the PLGA-PEGm NPs. Longer exposure time (120 min, 240 min) (Figure 11B, 11C) increased the inhibitory effect of all treatments and also resulted in a slower recovery of cell number. Whereas, a sharp recovery was witnessed in groups treated with the free drug, when the exposure time was short (30 min). Removal of drug/FA NP containing media, followed by a gentle wash could have also amplified the shock on the cells leading to higher inhibitory effects (cell loss) evident on day 1. Overall, FA NPs showed better sustained inhibitory effects when compared to free drug and PLGA-PEGm NPs.

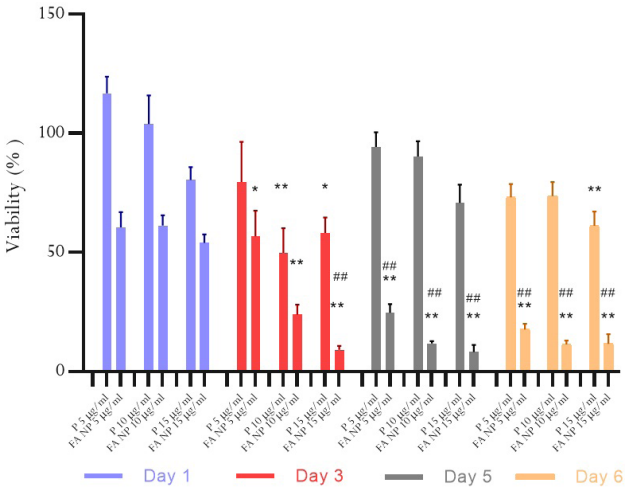

Figure 10. Effect of prednisolone and FA functionalized NPs on cell viability in U87 cells. Specific groups were treated with free drug prednisolone (P 5, 10, 15 µg/ml) and the FA NPs with equivalent drug concentrations (FA NP 5, 10, 15 µg/ml) for different time periods (Day 1, 3, 5, 6). The cell viability was determined as the percentage of control. Values are mean ± SD, (n=5) with * ($p < 0.05$), ** ($p<0.01$) vs control group; # ($p < 0.05$), ## ($p<0.01$) vs group of prednisolone in the same concentration.

Conclusion

To study the effect and efficacy of drug-loaded, FA functionalized-PLGA NPs on glioma cell lines, we formulated prednisolone encapsulated PLGA NPs with and without the FA moiety on the surface of the carriers and characterized them for size, zeta potential, and drug loading capacity. Similarly, Coumarin-6 (fluorescent molecule) loaded NPs were prepared to study the uptake efficiencies of the cell lines. FA NPs showed significantly higher uptake in C6, U87, and RAW 264.7 cells when compared to the free molecule used as a control and NPs without FA moiety, thereby indicating receptor-mediated endocytosis that facilitated a higher uptake of the FA functionalized formulations. The FA NPs proved better at attenuating the pro-inflammatory cytokines when compared to free drug. Cytotoxicity studies revealed

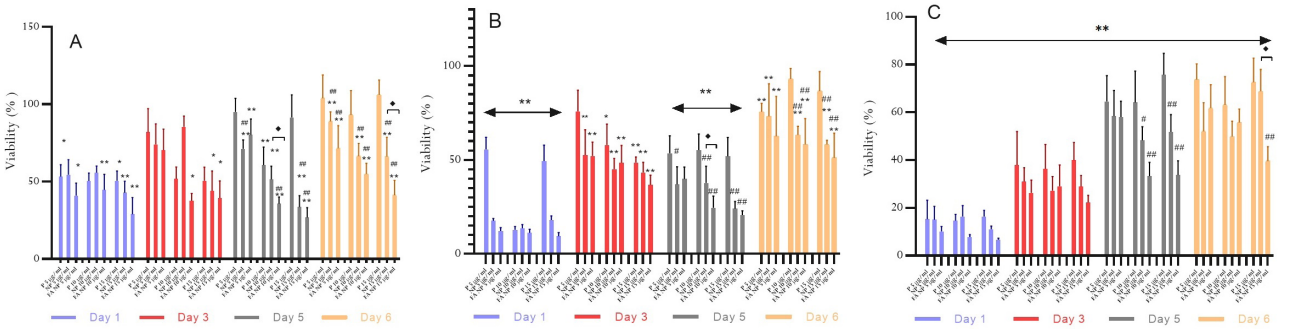

Figure 11. Effect of prednisolone and NPs on cell viability of C6. Specific groups were treated with free drug-prednisolone (P 5, 10, 15 µg/ml), NPs (PLGA-PEGm NPs 5, 10, 15 µg/ml) and FA NPs (FA NPs 5, 10, 15 µg/ml) with equivalent drug concentrations for 30 min (A), 120 min (B), and 240 min (C). The viability was determined after different time periods (Day 1, 3, 5, 6). Values are mean ± SD, (n=5) with * ($p < 0.05$), ** ($p<0.01$) vs control group; # ($p < 0.05$), ## ($p<0.01$) vs group of prednisolone in same concentration; ♦ ($p<0.05$) vs group without FA moiety.

that the inhibitory effects of the free drug on C6 cells diminished after 72 hours, whereas the effect was not so pronounced on U87 cells. FA NPs, on the other hand, showed significantly higher and sustained inhibitory effect on both the cell lines. The overall results indicated that FA NPs can be used as an effective drug delivery vehicle.

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Authors Contributions

The concept and design of this study was done by BRG and SA. Synthesis of NPs with and without folic acid moiety on the surface and characterization was done by SA. Acquisition of data and *in vitro* cell line analysis was made by SA and JV. Statistical analysis and manuscript writing was done by SA. The acquired data analysis and the written manuscript was supervised and approved by BRG. All authors read and gave approval of the final manuscript.

Conflict of Interest

Authors declare no conflict of interest.

Supplementary Data

Supplementary file is available on the journal's web site along with the published article.

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