



Enhanced and preferential internalization of lipid nanocapsules into human glioblastoma cells: effect of a surface-functionalizing NFL peptide

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Reatul Karim, Elise Lepeltier, Lucille Esnault, Pascal Pigeon, Laurent Lemaire, et al.. Enhanced and preferential internalization of lipid nanocapsules into human glioblastoma cells: effect of a surface-functionalizing NFL peptide. *Nanoscale*, 2018, 10, pp.13485-13501. 10.1039/C8NR02132E . hal-01817763

HAL Id: hal-01817763

<https://hal.sorbonne-universite.fr/hal-01817763>

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1 ENHANCED AND PREFERENTIAL
2 INTERNALIZATION OF LIPID
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5 SURFACE-FUNCTIONALIZING NFL PEPTIDE

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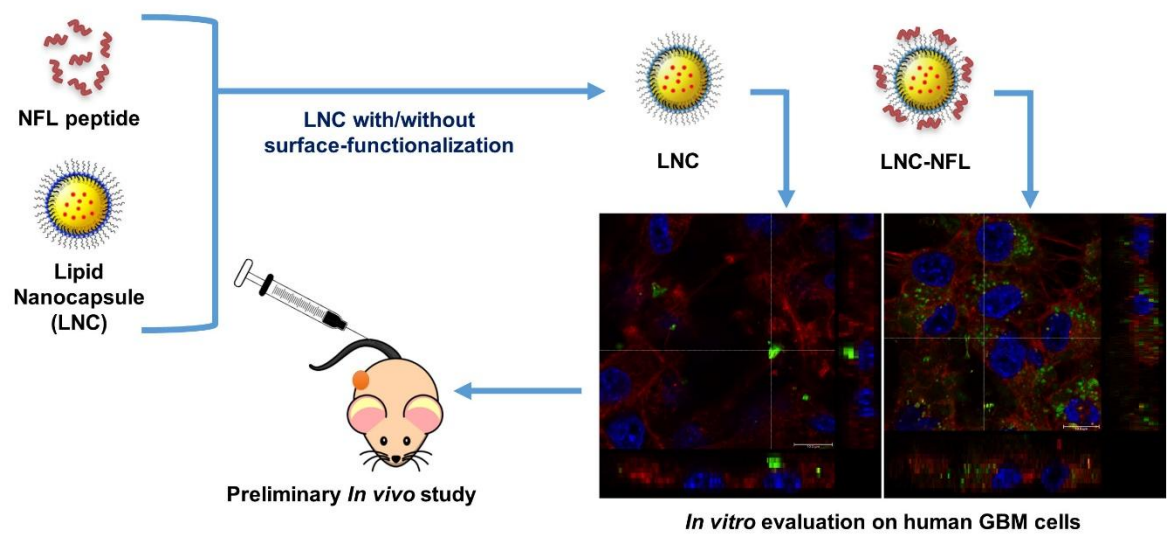
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ABSTRACT

Increasing intracellular drug concentration using nanocarriers can be a potential strategy to improve efficacy against glioblastoma (GBM). Here, the fluorescent-labelled NFL-TBS.40-63 peptide (fluoNFL) concentration on lipid nanocapsule (LNC) was studied to enhance nanovector internalization into human GBM cells. The LNC surface-functionalization with various fluoNFL concentrations was performed by adsorption. LNC size and surface charge altered gradually with increasing peptide concentration, but their complement protein consumption remained low. Desorption of fluoNFL from LNC surface was found to be slow. Furthermore, it was observed that the rate and extent of LNC internalization in the U87MG human glioblastoma cells were dependent on the surface-functionalizing fluoNFL concentration. In addition, we showed that the uptake of fluoNFL-functionalized LNCs was preferential towards U87MG cells compared to healthy human astrocytes. The fluoNFL-functionalized LNC internalization into the U87MG cells was energy-dependent and occurred possibly by macropinocytosis, clathrin-mediated and caveolin-mediated endocytosis. A new ferrocifen-type molecule (FcTriOH), as potent anticancer candidate, was then encapsulated in the LNCs and the functionalization improved its *in vitro* efficacy compared to other tested formulations against U87MG cells. In the preliminary study, on subcutaneous human GBM tumor model in nude mice, a significant reduction of relative tumor volume was observed at one week after the second intravenous injection with FcTriOH-loaded LNCs. These results showed that enhancing NFL peptide concentration on LNC surface is a promising approach for increased and preferential nanocarrier internalization into human GBM cells, and the FcTriOH-loaded LNCs are promising therapy approach for GBM.

Keywords: lipid nanocapsule, glioblastoma, ferrocifen, cell-penetrating peptide, NFL-TBS.40-63.

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Surface functionalized LNC showed a preferential internalization into GBM cells and demonstrated a significant decrease of the tumor volume.

INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most prevalent, and fatal primary brain tumors classified as the World Health Organization as a grade IV CNS tumor.¹ Although remarkable progress in diagnostic methods and treatment strategies has been achieved in the last few decades, the median survival only altered from 8.3 to 14.6 months over the last 60 years after present multimodal therapy (surgical resection followed by radiotherapy plus chemotherapy).²⁻⁴ Therefore, new therapeutic approaches for treatment of GBM are necessary.

Nanosized-drug delivery systems (NDDSs) have appeared as a promising strategy for drug delivery against cancer, including brain cancers. The NDDSs can have numerous beneficial characteristics *i.e.* prolonged blood circulation time, improved bioavailability of hydrophobic drugs, controlled drug release and site-targeted drug delivery.⁵ Moreover, long circulating nanocarriers with appropriate size may accumulate in malignant brain tumors after crossing the integrity impaired blood-brain barrier (BBB) by enhanced permeability and retention (EPR) effect, and improve survival time of animals.⁶ Among various nanocarriers, lipid nanocapsules (LNCs) have been reported in numerous literatures as promising NDDS for carrying hydrophobic drugs due to their characteristic oily core.⁷ One of the promising features of LNC formulation is its easy and organic solvent free preparation technique that can be easy to scale-up for future industrial purpose.⁸ LNCs were evaluated and showed promising *in vitro* and *in vivo* results against GBM in numerous studies.⁹⁻¹¹ A novel ferrocifen-type anticancer molecule, 4-ferrocenyl-5,5-bis(4-hydroxyphenyl)-pent-4-en-1-ol (FcTriOH) (Fig. 1), was encapsulated in the LNCs. The FcTriOH belongs to the newer hydroxypropyl series of ferrocifens which can convert into novel tetrahydrofuran-substituted quinone methide.¹²⁻¹³ Compared to the corresponding acyclic series ferrocifen, FcTriOH showed about 6-folds lower IC₅₀ (0.11 ± 0.02 μM) against

1 estrogen-receptor-negative breast cancer cells possibly due to the reduced polarity of the
2 intrinsically antiproliferative quinone methide (by cyclization of the hydroxyalkyl chain).¹² In
3 this study, the *in vitro* antiproliferative activity of the FcTriOH-loaded LNCs was thus studied on
4 another cell line : U87MG human GBM cells.

5 In order to enhance intracellular drug concentrations into GBM cells, the LNC surface can be
6 modified by adding various GBM-targeting ligands.¹⁴⁻¹⁷ A neurofilament light subunit derived 24
7 amino acid tubulin binding site peptide called NFL-TBS.40-63 (NFL) was reported to
8 preferentially be internalized into human, rat and mouse GBM cells compared to corresponding
9 healthy cells.¹⁸ This peptide was evaluated as potential GBM-targeting moiety on LNC surface in
10 rat or mouse GBM cell lines.¹⁹⁻²⁰ However, based on these studies and in order to be more
11 clinically relevant, it is necessary to evaluate the internalization capability of NFL-functionalized
12 LNCs into human GBM cells. Moreover, the concentration of the GBM targeting ligand should
13 also be optimized to achieve enhanced and preferential delivery to GBM cells. Therefore, the
14 aim of this study was to evaluate the effect of the LNC surface-adsorbed NFL concentration on
15 the nanovector internalization into human GBM cells, in order to achieve a preferential uptake
16 and avoid potential toxicity on healthy cells. The effect of NFL adsorption on the
17 physicochemical characteristics of LNC was studied. Additionally, impact of salt concentrations
18 on NFL-desorption from the LNC surface was studied. Moreover, influence of peptide-
19 adsorption on the complement protein consumption by the formulations was investigated. A
20 comparative cellular internalization kinetic study into human GBM cells with the various
21 developed LNCs was performed and confirmed by confocal microscopy study. Targeting ability
22 of the NFL-functionalized LNCs towards GBM cells was also assessed by comparing its uptake
23 into both GBM cells and astrocytes under identical conditions. Possible internalization pathway

of the functionalized-LNC into the human GBM cell line was assessed. Finally, a preliminary *in vivo* study was performed on an ectopic human GBM tumor model in mice in order to observe possible therapeutic effects after systemic delivery of the formulations.

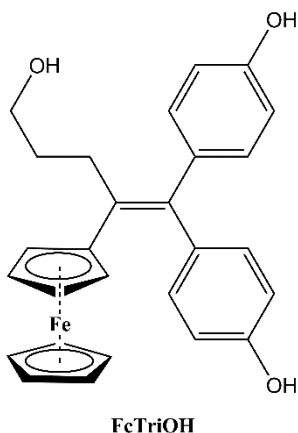


Figure 1: Structure of FcTriOH: 4-ferrocenyl-5,5-bis(4-hydroxyphenyl)-pent-4-en-1-ol.

RESULTS

Physicochemical characteristics of the nanocapsules

LNCs were prepared by phase-inversion technique as previously described.²¹ The surface of the LNCs was functionalized by adding 0.86 and 2.57 % (w/w) fluoNFL peptide in the LNC dispersion to prepare LNC-fluoNFL1 and LNC-fluoNFL2 respectively. The functionalization occurred by adsorption.^{19, 22} The initial molar concentration of NFL peptide for the LNC-fluoNFL1 was kept same as previously published literatures (1 mM)^{19, 22} whereas it was 3-folds higher in LNC-fluoNFL2. Final concentrations of the adsorbed fluoNFL (% w/w), determined by HPLC, on LNC-fluoNFL1 and LNC-fluoNFL2 was of 0.40 ± 0.01 % and 2.49 ± 0.01 % respectively.

Particle size, PDI and zeta potential of the different nanocapsule formulations determined by Dynamic Light Scattering (DLS) and laser Doppler electrophoresis are given in Table 1. The investigational conditions, *i.e.* LNC concentrations, sample viscosities, temperature and sample

conductivity were consistent among the measurements. The control LNC had a size of 57 ± 2 nm, PDI of 0.08 ± 0.01 and zeta potential of -2.2 ± 0.9 mV. LNC-fluoNFL1 had a size of 61 ± 1 nm, PDI of 0.12 ± 0.02 and zeta potential of 0.5 ± 0.7 mV (Table 1). Additionally, LNC-fluoNFL2 had the highest values among the three formulations *i.e.* size of 64 ± 1 nm, PDI of 0.15 ± 0.02 and zeta potential of 4.9 ± 1.5 mV.

Encapsulation of FcTriOH in LNC (LNC-FcTriOH) significantly ($p < 0.001$) reduced the particle size to 50 ± 2 nm, compared to control LNC. Drug-loading of LNC-FcTriOH was 2.67 % (w/w) with an encapsulation efficiency of 99.8 ± 2.3 %. After fluoNFL adsorption, the size of LNC-FcTriOH-fluoNFL2 was 58 ± 1 nm which was significantly ($p < 0.001$) larger compared to LNC-FcTriOH. PDI and zeta potential was not altered after FcTriOH encapsulation with/without fluoNFL functionalization compared to respective unloaded LNCs.

Table 1: Physicochemical characteristics of the nanocapsules

Formulation	Size (nm)	PDI	Zeta potential (mV)
Control LNC	57 ± 2	0.08 ± 0.01	-2.2 ± 0.9
LNC -fluoNFL1	$61 \pm 1^{**}$	$0.12 \pm 0.02^{**}$	$0.5 \pm 0.7^{**}$
LNC-fluoNFL2	$64 \pm 1^{***}$	$0.15 \pm 0.02^{***}$	$4.9 \pm 1.5^{***}$
LNC-FcTriOH	$50 \pm 2^{***}$	0.06 ± 0.02	-2.3 ± 1.3
LNC-FcTriOH-fluoNFL2	58 ± 1	$0.15 \pm 0.08^*$	$3.4 \pm 0.6^{***}$

(Oneway ANOVA with Dunnett's post-test. $p < 0.05$ is denoted by (*), $p < 0.01$ by (**) and $p < 0.001$ by (***), $n=6$)

Interaction between LNCs and fluoNFL

The interaction between the LNC surface and NFL peptide in a formulation equivalent to LNC-fluoNFL1 was described previously.²² To understand the interaction between the LNC surface and fluoNFL, the LNC-fluoNFL2 and LNC as control were incubated for 30 min in UPW and different concentrations of NaCl or Tris buffer pH 7.4. Subsequently, their size was measured in DLS (Fig. 2). The size of LNC-fluoNFL2 remained significantly different compared to control LNCs as NaCl concentration was increased up to 1 mM. However, as concentration of Tris buffer increased above 0.05 M, the size of LNC-fluoNFL2 was reduced and its significant difference compared to control LNCs was lost.

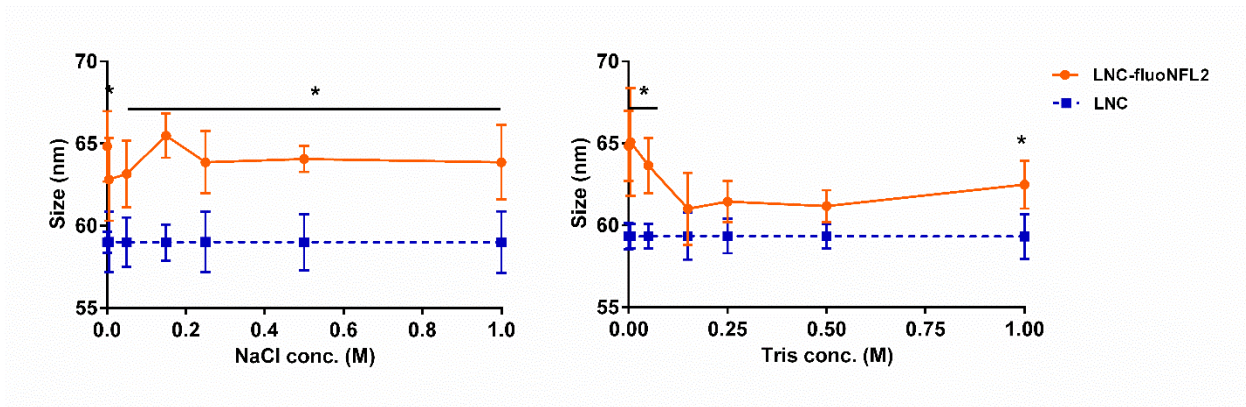


Figure 2: Mean particle sizes of control LNCs and LNC-fluoNFL2 in various concentrations of NaCl and Tris buffer (t-test. $p < 0.05$ is denoted by (*), $p < 0.01$ by (**) and $p < 0.001$ by (***), $n = 3$).

Moreover, LNC-fluoNFL2 was dialyzed against 0.05 M Tris buffer at 37 °C and 75 rpm using a dialysis bag having MWCO 100 kD. At various time points, the amount of fluoNFL in the receiver chamber (desorbed from the formulation) was quantified by HPLC. As control, the peptide solution was dialyzed and quantified in the receiver chamber. The control peptide solution reached the receiver chamber very quickly and more than 90 % of the peptide was

recovered by 1 h. However, a slow and gradual desorption of the peptide was observed (Fig. 3) from the LNC surface and only 6 % peptide desorption occurred by 30 min and reached 33 % by 6 h.

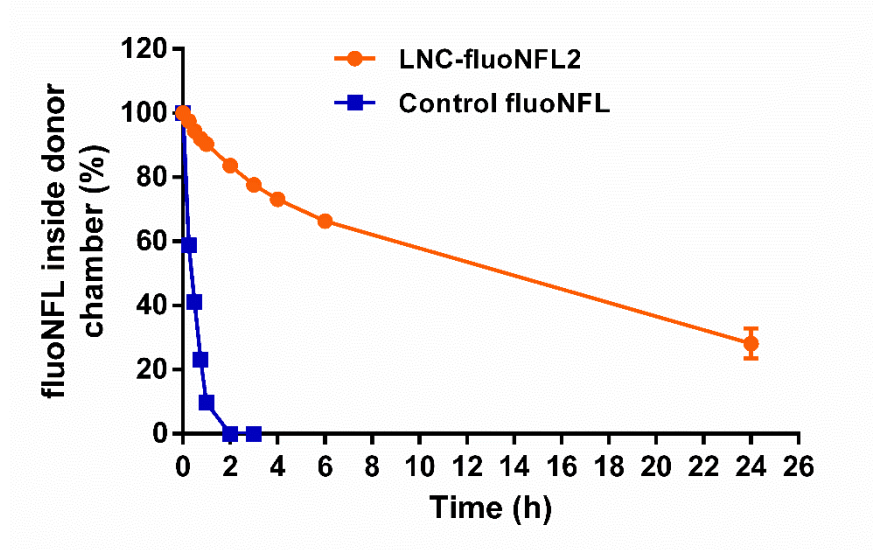
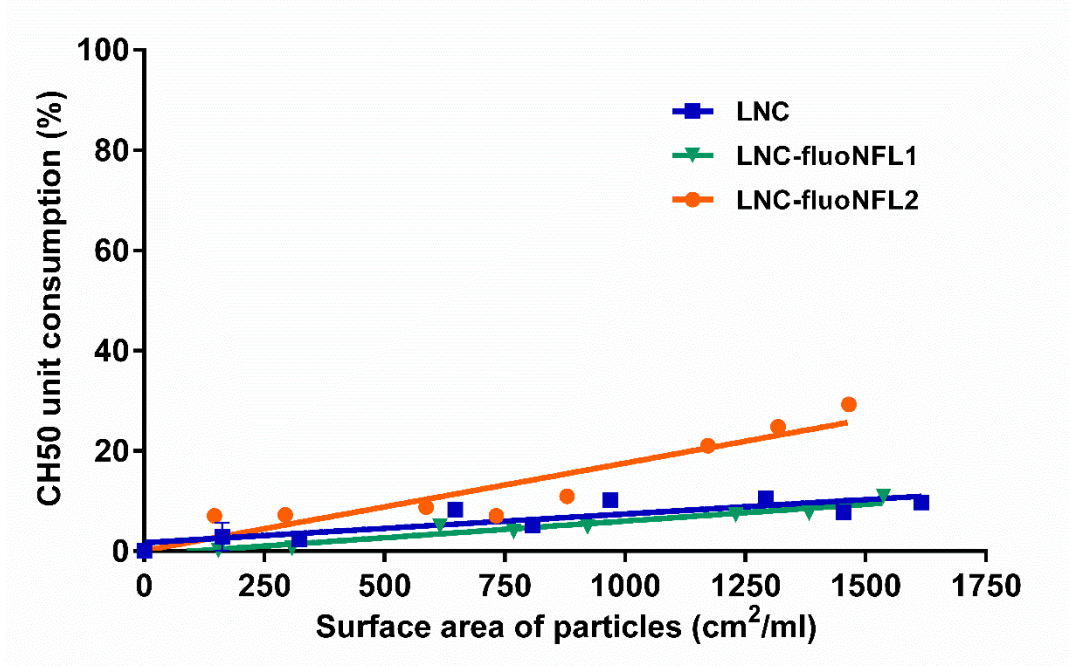


Figure 3: FluoNFL desorption kinetics from LNC-fluoNFL2 surface in 0.05 M Tris buffer pH 7.4 at 37 °C and 75 rpm.

Complement consumption by the nanocapsules

Complement consumption by the control LNCs and the fluoNFL-functionalized LNCs was assessed by the CH50 assay. The particle concentration in the control LNCs was quantified by NTA and was used to calculate surface area of the LNC formulations. The percentage of CH50 unit consumptions by the control LNCs, and the peptide functionalized LNCs were plotted against surface area of the nanocapsules in 1 mL of NHS (Fig. 4). The complement consumption by all three nanocapsules increased as surface area of the nanovectors increased per mL of NHS. The percentage of CH50 unit consumption by control LNCs and LNC-fluoNFL1 was similar and reached only 9.8 and 7.6 % respectively at around 700 cm²/mL NHS. The complement

1 consumption by LNC-fluoNFL2 was slightly higher and reached 21.0 % at the same surface
2 area.



3
4 **Figure 4:** Complement consumption at 37 °C by control LNCs, LNC-fluoNFL1 and LNC-
5 fluoNFL2.

6 **Effect of surface-functionalizing fluoNFL concentration on LNC internalization into**
7 **human GBM cells**

8 LNC concentration used for the cellular uptake studies was 1.23 mg/mL (corresponds to 11.1
9 μ M NFL in LNC-fluoNFL2), which was primarily selected based on previously described safe
10 concentrations of LNCs and NFL peptide.^{19, 22-23} Moreover, results of the MTS assays on
11 U87MG cells treated with the peptide solutions or blank LNCs showed that cell viability did not
12 decrease at these concentrations (Supporting Information: Fig. S1 and S2). The U87MG cells
13 were treated with DiA-labelled LNCs (LNC-DiA, LNC-DiA-fluoNFL1 and LNC-DiA-
14 fluoNFL2) for 30 min, 1 h, 6 h and 24 h to assess their cellular internalization at each time point

(Fig. 5). For each formulation, the cellular uptake increased as time was increased, showing the time dependency of the cell internalization. The internalization of LNC-DiA was of 0.2, 0.8, 2.3 and 11.8 % after 30 min, 1 h, 6 h and 24 h respectively. LNC-DiA-fluoNFL1 uptake was 1.2, 2.7, 46.5 and 81.9 % after 30 min, 1 h, 6 h and 24 h respectively; whereas it was 8.4, 16.6, 72.4 and 86.2 % for LNC-DiA-fluoNFL2. At each time point, the cellular internalization of LNC-DiA-fluoNFL2 was significantly higher compared to LNC-DiA-fluoNFL1 and LNC-DiA, whereas uptake of LNC-DiA-fluoNFL1 was significantly higher compared to LNC-DiA.

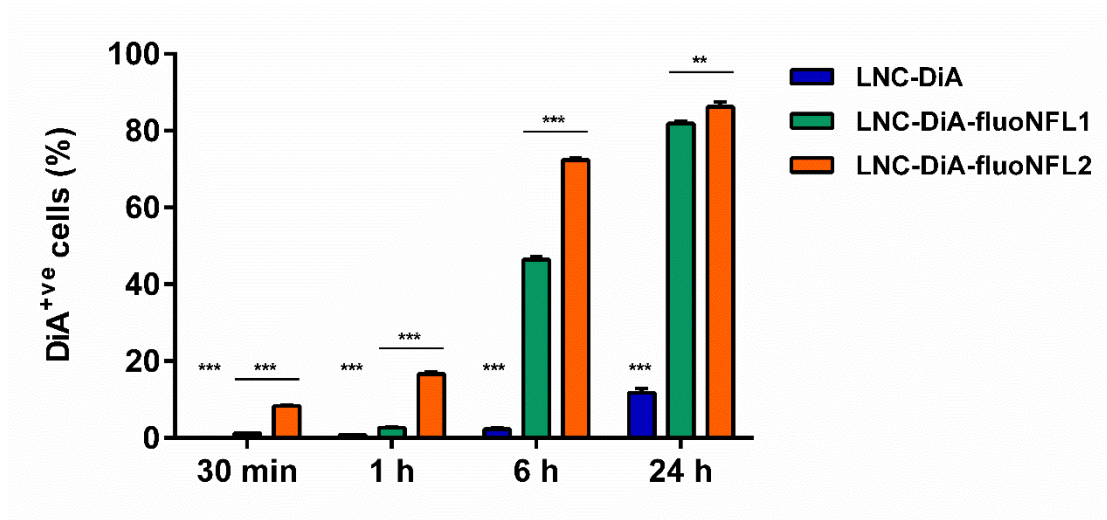


Figure 5. Enhancement of LNC internalization at 37 °C into U87MG cells with increasing concentration of the fluoNFL peptide on LNC surface. The cells were incubated with 1.23 mg/mL of LNC-DiA, LNC-DiA-NFL1 and LNC-DiA-fluoNFL2 for 30 min, 1 h, 6 h and 24 h. Twenty thousand events per sample were analyzed and percentages of DiA^{+ve} cells were measured. The experiments were performed in triplicate. Statistical analysis was performed with one-way ANOVA with Tukey post-hoc test (p <0.05 is denoted by (*), p <0.01 by (**)) and p <0.001 by (***), n=3).

Moreover, to investigate the necessity of the peptide adsorption on LNC (during the formulation of LNC-fluoNFL2) to enhance its cellular uptake, DiA-labelled LNC and fluoNFL (at same peptide concentration as LNC-DiA-fluoNFL2) were mixed to prepare ‘LNC-DiA & fluoNFL imm. mix.’ and the cells were treated immediately for 1 h at 37 °C. The uptake of the immediate mixture was significantly lower (3.9-folds) compared to LNC-DiA-fluoNFL2, but slightly higher compared to LNC-DiA (Fig. 6).

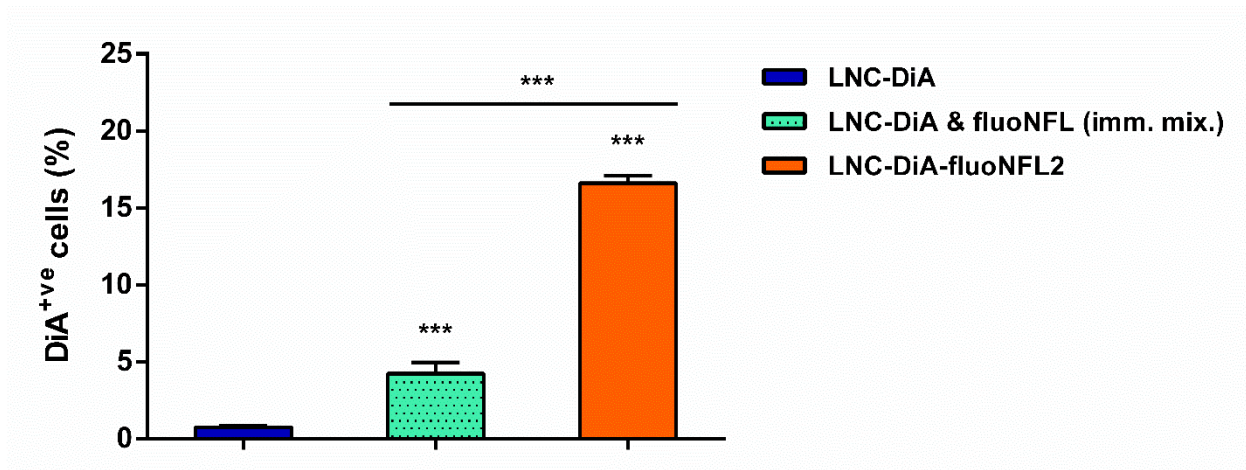
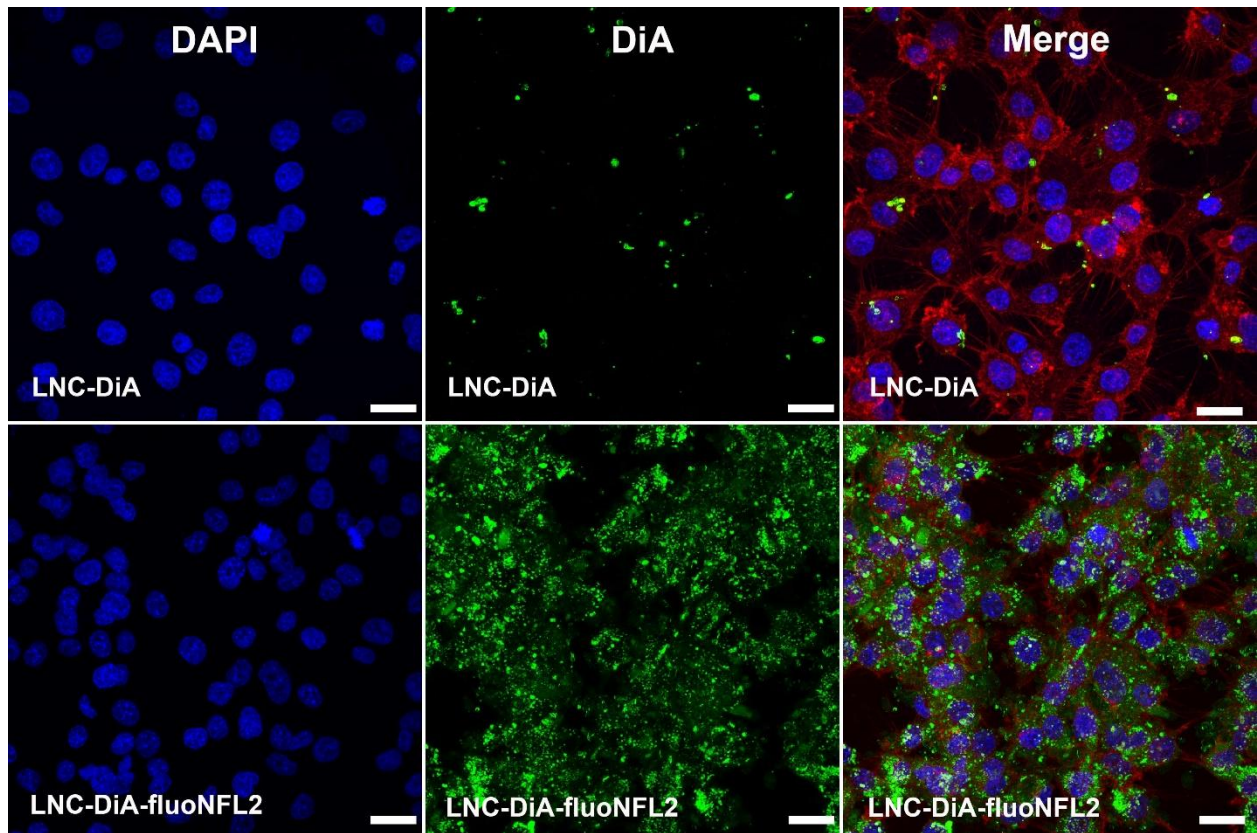


Figure 6: Internalization of LNC-DiA, immediate mixture of LNC-DiA and fluoNFL2, and LNC-DiA-fluoNFL2 into U87MG cells at 37 °C after 1 h. The cells were incubated with 1.23 mg/mL of LNC-DiA, ‘LNC-DiA & fluoNFL imm. mix.’ and LNC-DiA-fluoNFL2 for 1 h. Twenty thousand events per sample were analyzed and percentage of DiA⁺ve cells was measured. The experiments were performed in triplicate. Statistical analysis was performed with oneway ANOVA with Tukey post-hoc test (p <0.05 is denoted by (*), p <0.01 by (**) and p <0.001 by (***), n=3).

Additionally, the higher cellular internalization of LNC-fluoNFL2 compared to control LNCs was visualized by confocal microscopy (Fig. 7). The cells were first treated by LNC-DiA (green dye) and LNC-DiA-fluoNFL2 (green dye) for 6 h, followed by staining of their nuclei (DAPI

1 staining: blue) and cytoskeleton (phalloidin-TRITC staining: red) for capturing confocal images.
 2 The DiA signal was much higher for the fluoNFL-functionalized LNCs compared to control
 3 LNCs, and almost each cell had DiA signal in its cytoplasm.



4
 5 **Figure 7.** Representative confocal microscopy images of enhanced LNC internalization into
 6 U87MG cells due to LNC surface-functionalizing fluoNFL peptide. Cells were treated at 37 °C
 7 for 6 h with 1.23 mg/mL of LNC-DiA and LNC-DiA-fluoNFL2. Blue is DAPI staining (nuclei),
 8 green is DiA (LNC) and red is phalloidin-TRITC staining (F-actin, cytoskeleton). White bar = 20
 9 μm. Supporting information: Fig. S3.

10 To visualize if the LNCs were on the cell surface or inside the cytoplasm, orthogonal sections
 11 of the stacked images were analyzed (Fig. 8). Indeed, nearly all the fluorescence was observed
 12 inside the cytoplasm of the cells and each cell had internalized lots of NFL functionalized

nanocapsules. In comparison, the control LNCs were localized predominantly on the cell surface rather than inside the cytoplasm.

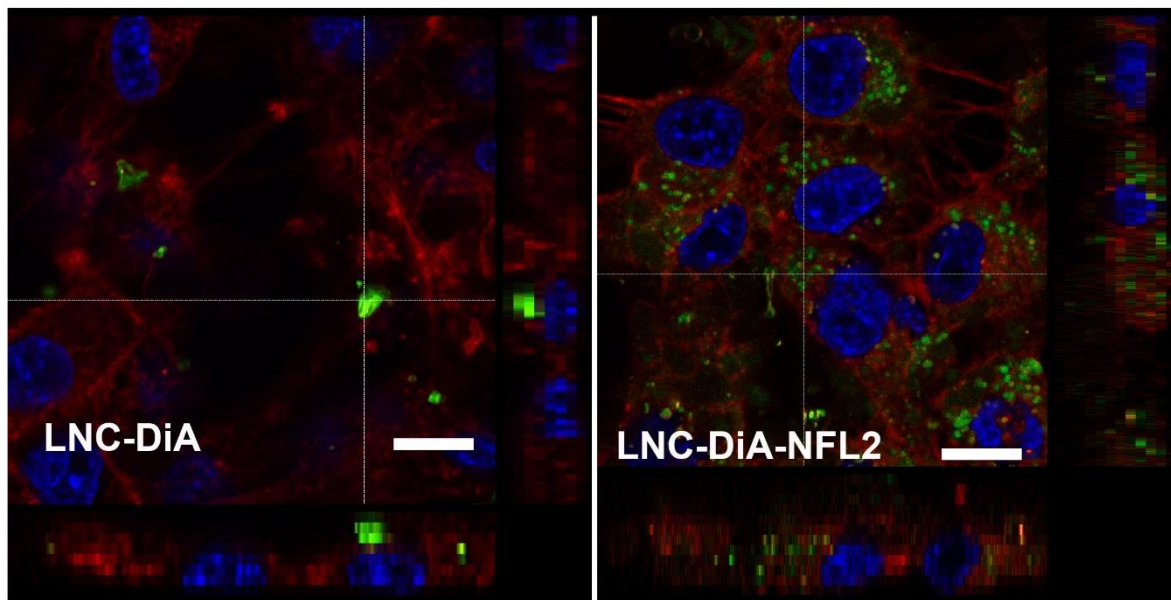


Figure 8. Orthogonal sections of stacked confocal microscopy images showing LNC internalization into U87MG cells. Majority of LNC-DiA-NFL2 was localized into cell cytoplasm, whereas LNC-DiA was chiefly localized on cell surface. The cells were treated at 37 °C for 6 h with 1.23 mg/mL of LNC-DiA and LNC-DiA-NFL2. Blue is DAPI staining (nuclei), green is DiA (LNC) and red is phalloidin-TRITC staining (F-actin, cytoskeleton). White bar = 10 μ m.

Preferential accumulation of fluoNFL functionalized lipid nanocapsules in human GBM cells compared to normal human astrocytes

To inspect the targeting capability of the fluoNFL-functionalized LNC towards human GBM cells, internalization of DiA loaded LNC and LNC-fluoNFL2 into NHA were measured and compared with the uptake in U87MG cells. The internalization of LNCs was significantly higher in Normal Human Astrocytes (NHA) compared to U87MG cells at 1 h and 6 h (Fig. 9). Surface-functionalization with the fluoNFL peptide significantly enhanced the uptake of LNCs in NHA

by 5.2-folds and 3.5-folds at 1 h and 6 h respectively, compared to control LNCs (LNC-DiA) (Fig. S4). In contrast, LNC functionalization with fluoNFL enhanced the LNC uptake into U87MG cells by 21.6-folds and 31.5-folds at 1 h and 6h respectively, compared to control LNCs. Although there was no significant difference of LNC-DiA-fluoNFL2 internalization in NHA and U87MG cells at 1 h, the uptake was significantly higher (4.4-folds) in the GBM cells by 6 h.

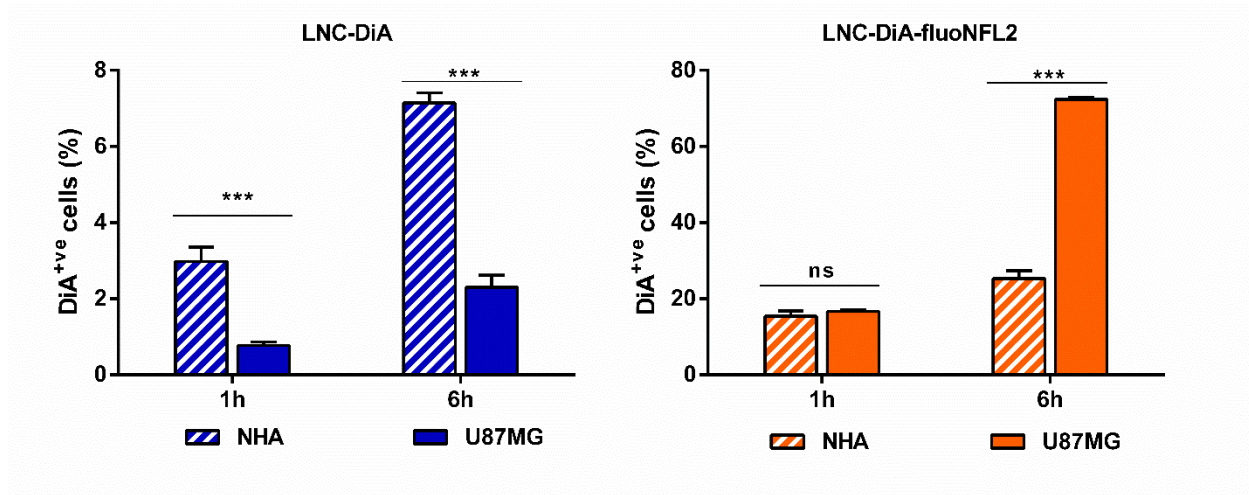


Figure 9: Higher LNC-DiA internalization into NHA compared to U87MG cells, whereas LNC-DiA-fluoNFL2 was internalized preferentially into U87MG cells compared to NHA. The cells were incubated with 1.23 mg/mL of LNC-DiA or LNC-DiA-fluoNFL2 for 1 h and 6 h. Twenty thousand events per sample were analyzed and percentages of DiA⁺ cells were measured. The experiments were performed in triplicate. Statistical analysis was performed with t-test ($p < 0.05$ is denoted by (*), $p < 0.01$ by (**), and $p < 0.001$ by (***), $n=3$). Supporting Information: Fig. S4.

Mechanisms of fluoNFL-functionalized lipid nanocapsule internalization in U87MG human glioblastoma cell

To evaluate the possible mechanism of LNC-fluoNFL2 internalization in U87MG cells, the cells were treated with the DiA-labelled nanocapsules in different energetic conditions *i.e.* at 4°C and ATP-depleted conditions for 1 h and 6 h (Fig. 10a). At 4°C, the internalization of LNC-DiA-

fluoNFL2 was almost completely stopped both at 1 h and 6 h and therefore the alteration was significant compared to the normal conditions (n.c.). In ATP-depleted conditions, LNC-DiA-fluoNFL2 uptake was near 0 % after 1 h, but increased to about 25 % of the n.c after 6 h. At 1 h, the LNC-DiA-fluoNFL2 uptake was similar in both conditions (4 °C and ATP-depleted), but significantly different after 6 h. Internalization of fluoNFL solution was significantly and similarly reduced at 4°C and in ATP-depleted condition at 1 h compared to n.c. (Fig. 10b). Therefore, LNC-fluoNFL2 uptake in U87MG cells was temperature and energy-dependent process, similarly to fluoNFL as previously reported.¹⁸

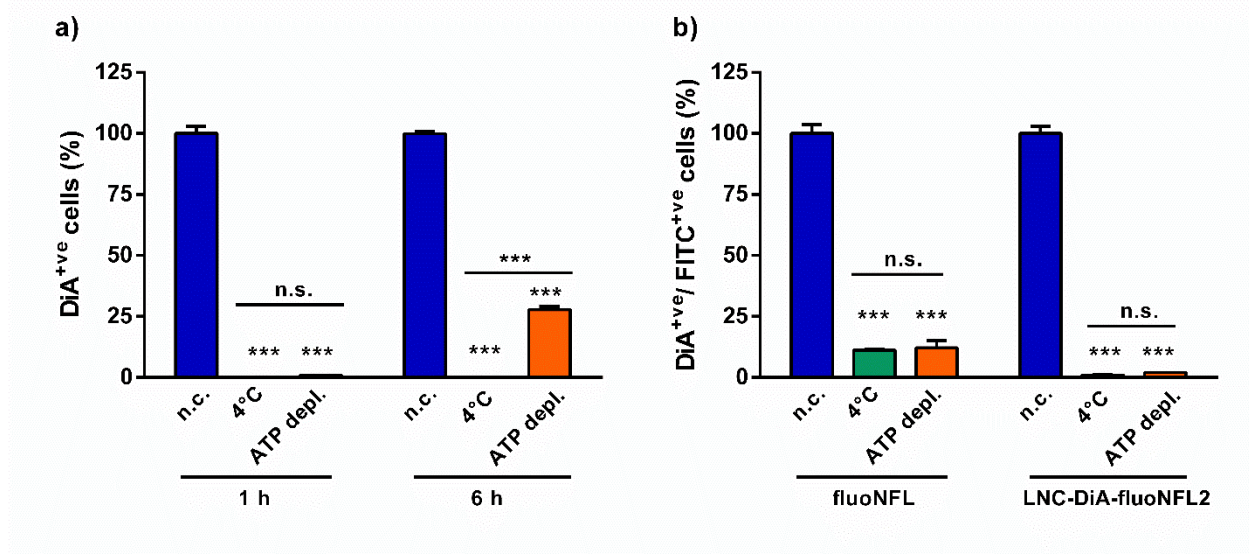


Figure 10: a) LNC-DiA-fluoNFL2 internalization into U87MG cells at different energetic conditions. The cells were incubated with 1.23 mg/mL of LNC-DiA-fluoNFL2 for 1 h and 6 h at 37 °C (n.c.), 4°C and ATP-depleted conditions. b) Comparison of internalization of fluoNFL and LNC-DiA-fluoNFL2 in U87MG cells at different energetic conditions at 1 h. Twenty thousand events per sample were analyzed and percentages of DiA⁺ve cells (for LNC-DiA-fluoNFL2) or FAM⁺ve cells (for fluoNFL) were measured. The experiments were performed in triplicate.

Statistical analysis was performed with oneway ANOVA with Tukey post-hoc test ($p < 0.05$ is denoted by (*), $p < 0.01$ by (**) and $p < 0.001$ by (***), $n=3$).

To further evaluate the possible uptake pathway(s) of the peptide-functionalized LNCs, exclusion of particular endocytosis mechanisms was achieved by using inhibitors of the foremost endocytosis pathways. The cells were pretreated for 30 minutes with different inhibitors followed by 1 h treatment with the LNC-DiA-NFL2. LNC uptake was significantly inhibited in presence of each of these inhibitors (Fig. 11a). LNC-DiA-NFL2 internalization was the lowest in presence of DAM, followed by CP, M β CD and PMA. A strong correlation between fluoNFL internalization and LNC-DiA-fluoNFL2 uptake was observed (Fig. 11b). Like the functionalized-LNC, fluoNFL uptake was most strongly inhibited by DAM, followed by similar inhibition in presence of CP and M β CD, and lastly PMA.

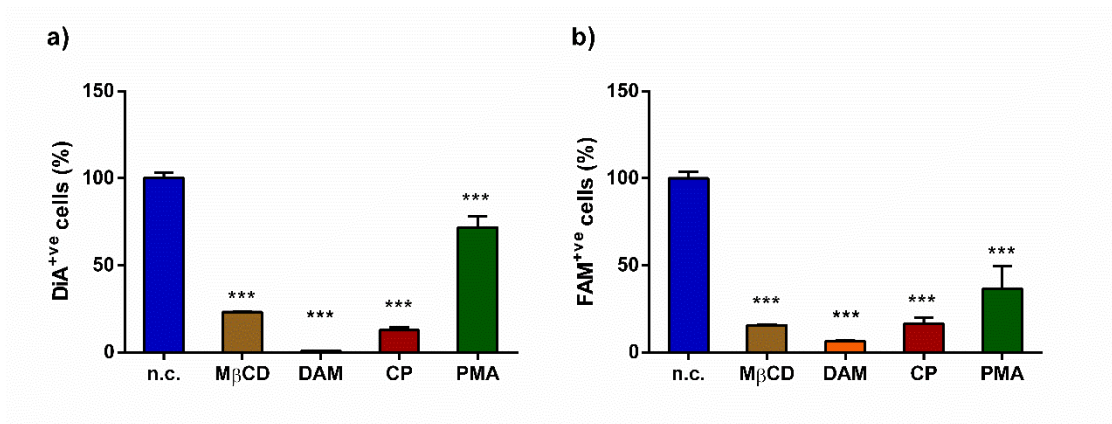


Figure 11: Internalization of LNC-DiA-fluoNFL2 (a) and fluoNFL (b) into U87MG cells at 37 °C (n.c.) after 30 min pretreatment with various inhibitors (M β CD, DAM, CP and PMA) followed by 1 h incubation with the nanocapsule (1.23 mg/mL) or the peptide solution (equivalent fluoNFL concentration of LNC-DiA-fluoNFL2). Twenty thousand events per sample were analyzed and percentages of DiA⁺ve cells (for LNC-DiA-fluoNFL2) or FAM⁺ve cells (for fluoNFL) were measured. The experiments were performed in triplicate. Statistical analysis was

performed with oneway ANOVA with Dunnett's post-hoc test ($p < 0.05$ is denoted by (*), $p < 0.01$ by (**) and $p < 0.001$ by (***), $n=3$).

***In vitro* efficacy on U87MG cells**

To evaluate the *in vitro* efficacy of FcTriOH loaded LNC formulations, cell viability was evaluated by MTS assay after 72 h of treatment with the formulations (Fig. 12). The IC_{50} of FcTriOH solution was of 1.31 μM which was slightly reduced to 1.05 μM when the drug was loaded in LNC. However, the peptide-functionalized LNC-FcTriOH-NFL2 had the lowest IC_{50} of 0.46 μM , which was 2.8-folds and 2.3-folds lower compared to the drug solution and the drug-loaded non-functionalized LNCs (LNC-FcTriOH). The blank LNCs showed toxicity at much higher concentrations (IC_{50} 1.42 mg/mL) compared to the drug-loaded LNCs (Supporting information: Fig. S1). The fluoNFL solution did not show any toxicity in the tested concentrations (Supporting information: Fig. S2).

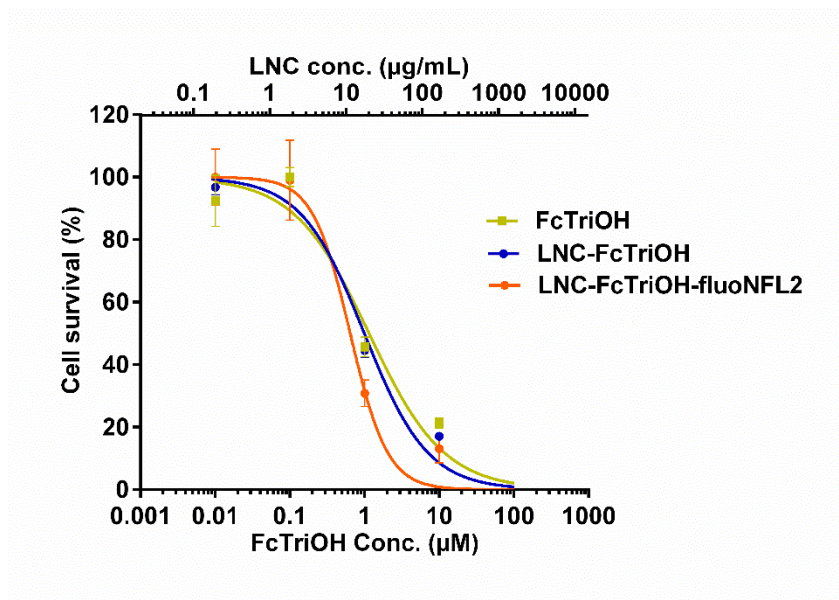


Figure 12. Survival percentages of U87MG cells after 72 h treatment with various concentrations of LNCs (LNC, LNC-FcTriOH and LNC-FcTriOH-NFL2) and FcTriOH solution.

Preliminary *in vivo* study in ectopic tumor xenograft model

Nude NMRI mice were subcutaneously inoculated with human U87MG cells to acquire preliminary knowledge of possible tumor reduction efficacy and toxicity of the developed formulations after i.v. administration. After 7 days of cell implantation, the average tumor volume was around 70 mm³ and the animals were divided into five groups (saline, LNC-blank, LNC-blank-fluoNFL2, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2) and injected intravenously with 70 µL of treatments (20 mg FcTriOH per kg body weight, or 822.4 mg LNCs with/without 21.5 mg peptide per kg of body weight) on day 7 and day 10 (Fig. 13). The relative tumor volume of the saline and the LNC-blank treated mice gradually increased from day 7 until the end of the study, whereas it was stable until day 17 and then increased for LNC-blank-fluoNFL2 treated group. For the FcTriOH treated groups (LNC-FcTriOH and LNC-FcTriOH-fluoNFL2), relative tumor volume gradually decreased up to day 17, remained smaller than their initial volume (at the first treatment injection day) up to day 22, and then increased gradually. Compared to saline treated group, the relative tumor growth for LNC-FcTriOH and LNC-FcTriOH-fluoNFL2 treated groups were significantly lower (40.1 and 44.2 % lower respectively) at day 17 of the study (Fig. 13). This significant difference was maintained up to day 22 of the study, and was absent afterwards.

None of the mice showed any immediate or delayed behavioral signs of pain or toxicity after the treatments were administered. Moreover, they were growing gradually as evident from their relative weight which increased about 20 % at the end of the study period (day=50): Fig. 13.

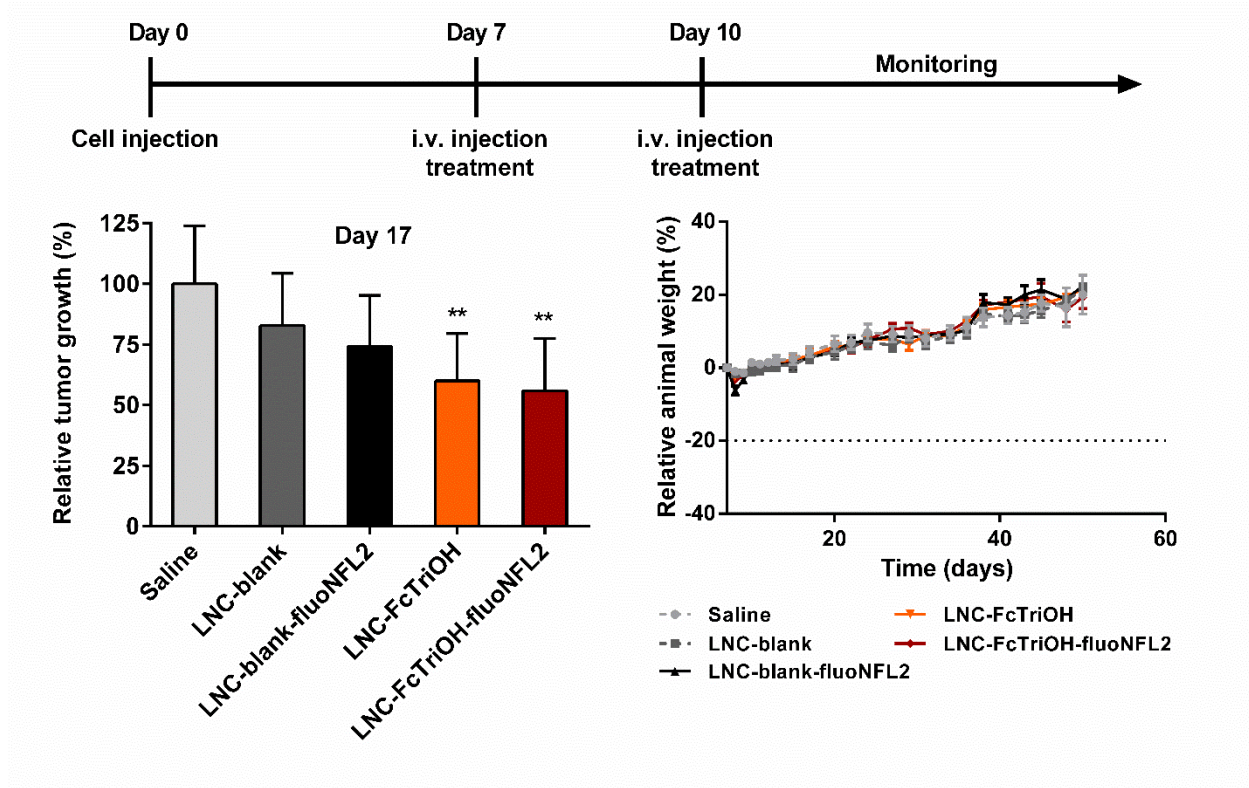


Figure 13: Relative tumor growth (on day 17) and relative animal weight of subcutaneous U87MG human glioblastoma tumor bearing mice. Each mouse was injected with 2×10^6 cells (in 50 μ L PBS) in the right flank on day 0 of the study. As the average tumor volume reached about 70 mm³ after one week, the mice received their treatment (equivalent to 20 mg FcTriOH per kg of body weight) by i.v. injections on day 7 and day 10. Mouse weight, behavior and tumor volume was followed regularly. Statistical analysis was performed with one-way ANOVA with Dunnett post-hoc test ($p < 0.05$ is denoted by (*), $p < 0.01$ by (**), $p < 0.001$ by (***), $n=7$ for saline, $n=8$ for other groups)

DISCUSSION

The aim of this study was to evaluate the effect of surface-functionalizing fluoNFL peptide concentration on LNC internalization into human GBM cells, in order to improve their efficacy as a drug delivery system for GBM. LNCs are promising nanovectors for carrying hydrophobic

1 anticancer molecules and has been used in numerous preclinical studies using various GBM
2 tumor models and different administration routes.^{9, 24-25} However, GBM tumors are known to
3 develop resistance to such treatments.²⁶ Therefore, enhancement of cellular internalization of
4 LNCs into GBM cells by functionalizing their surface can be a promising approach to improve
5 therapeutic efficacy. The NFL peptide was reported to preferentially enter in GBM cells from
6 diverse origins (human, rat and mouse) compared to corresponding healthy cerebral cells, and
7 reduced cell viability at $\geq 100 \mu\text{M}$.¹⁸ Moreover, treatment with NFL-functionalized LNCs
8 encapsulating an anticancer molecule (e.g. ferrocifens) which has a different mechanism of
9 action than the peptide may improve therapeutic outcomes by limiting resistance development by
10 GBM cells. The potential of this peptide as a GBM-targeting ligand to functionalize LNC surface
11 was investigated by Balzeau *et al.*, and increased cellular uptake of LNCs into mouse GBM
12 cells.¹⁹ In this study, the capability of NFL-peptide to act as a targeting ligand for the U87MG
13 human GBM cells and the effect of surface-functionalizing NFL concentration on cellular
14 internalization of LNCs were evaluated. Finally, the efficacy of NFL-LNCs encapsulating a
15 ferrocifen-type anticancer molecule, FcTriOH, was tested.

16 Covalent coupling of the peptide on distal end of PEG chains was not performed as it
17 diminished the GBM targeting capability of NFL.¹⁹ Therefore, surface-functionalization of LNCs
18 was performed by simply adsorbing different amounts of the peptide onto LNC surface over 24 h
19 period. As size, zeta potential and surface coating can profoundly impact on the *in vivo* fate of
20 the nanovectors,²⁷ these properties of the developed LNCs were characterized (Table 1). The
21 particle size of the control LNCs was $57 \pm 2 \text{ nm}$, whereas diameter of the LNC-fluoNFL1 (NFL
22 1 mM) and LNC-fluoNFL2 (NFL 3 mM) were about 4 nm and 7 nm larger respectively
23 signifying a potential higher amount of fluoNFL adsorbed to the surface. Similarly, peptide

1 adsorption increased the zeta potential of the LNC-fluoNFL1 and LNC-fluoNFL2 by +2 and +7
2 mV respectively compared to control LNC. This variation of surface charge can be explained by
3 the net positive charge of the NFL peptide at physiological pH.²⁸ The changes in size and zeta
4 potential for LNC-fluoNFL1 were similar to the one reported by Carradori *et al.*²² Moreover, the
5 LNC size after fluoNFL adsorption was well below 100 nm which can be beneficial for diffusion
6 in the cerebral extracellular space.^{9, 29} The PDI of all three formulations were less than 0.2,
7 therefore they can be considered as monodispersed. The concentration of the LNC-adsorbed
8 fluoNFL was quantified indirectly by measuring the free peptide concentration after separating
9 them using centrifugal filters with MWCO 100 kD. The concentration of LNC-adsorbed peptide
10 in LNC-fluoNFL1 was 0.40 % w/w which was in correspondence with the concentration
11 reported by Balzeau *et al.* and Carradori *et al.*^{19, 22} The concentration of LNC adsorbed fluoNFL
12 in LNC-fluoNFL2 was 2.49 % (w/w) which was about 6-folds higher compared to LNC-
13 fluoNFL1 although the concentration of peptide initially added was only 3-folds higher. The
14 number of peptide molecules per LNC particle can be calculated from the particle concentration
15 obtained by NTA and the adsorbed NFL concentration quantified by HPLC. About 240 and 1530
16 peptides were adsorbed per LNC particle in LNC-fluoNFL1 and LNC-fluoNFL2 respectively.
17 Torchilin *et al.* determined the number of TAT peptides on 200 nm liposomes by radiolabeling
18 the peptide and measuring liposome-associated radioactivity. About 500 peptides per liposome
19 was calculated,³⁰ which was included in the range of fluoNFL chains used in our formulations.
20 About 0.016 and 0.102 fluoNFL molecules will be present per nm² surface area in LNC-
21 fluoNFL1 and LNC-fluoNFL2 respectively, whereas up to 5.4 short chain PEG molecules
22 (comes from Kolliphor HS15) per nm² surface area can be present in average. The exact reason
23 for such high percentage of adsorption is unknown. It can be hypothesized that 3-folds increased

1 peptide concentration during the 24 h adsorption step possibly increased the likelihood of
2 collision and amplified the LNC-peptide and peptide-peptide interactions, which in combination
3 may have resulted in the high percentage of NFL adsorption. Physical entanglement between
4 adjacent peptide molecules might also occur in presence of LNCs at this concentration, resulting
5 restrained peptide movement and increased adsorption.³¹ Moreover, the aqueous dispersion of
6 LNCs became a gel after adsorption by an initial concentration of 4 mM fluoNFL (therefore NFL
7 concentrations above 3 mM were not tested). This also indicated that the peptide-LNC mixture
8 may start to form a rigid network at high peptide concentrations. Self-assembly of peptides has
9 been described to form hydrogels in the literature.³² Alteration in environmental conditions (*e.g.*
10 pH and ionic strength) can trigger interaction among peptide chains resulting physical cross-
11 linking and filament growth to form viscoelastic solids.³³ Addition of LNC dispersion may alter
12 such environmental conditions of the peptide solution and result formation of semi-solids.

13 Balzeau *et al.* has reported that the NFL interacts with the polar PEG chains of the Kolliphor¹⁹
14 whereas Carradori *et al.* suggested that the interaction was possibly by a combination of
15 electrostatic forces and other weak forces *i.e.* Van der Waal's forces and hydrophobic forces.²²
16 We evaluated the effect of NaCl and Tris buffer concentration on LNC-fluoNFL2 size by
17 incubation with different concentrations of these solutions and subsequently measuring their
18 diameter in DLS (Fig. 2). Contrasting to previous studies, the significant difference of
19 nanocapsule diameter compared to control LNCs was maintained nearly throughout the NaCl
20 concentration range. However, Tris buffer impacted more the size of LNC-fluoNFL2 compared
21 to NaCl, and no significant difference of particle size was observed above 0.05 M concentration.
22 It can be hypothesized that the possible self-entanglement of the peptide in LNC-fluoNFL2
23 involves more inter-chain interactions (*e.g.* hydrogen bond, hydrophobic forces and/or Van der

Waal's forces) and therefore resisted the impact of high NaCl concentrations, but loses its significant size difference with control LNCs in higher Tris concentrations. To evaluate if the fluoNFL will be rapidly removed from the LNC surface after dilution, LNC-fluoNFL2 was dialyzed (MWCO 100 kD dialysis bag) against 1x Tris buffer solution at 37 °C and 75 rpm. Free peptide concentration was quantified from the receiver compartment by HPLC. Desorption of the fluoNFL from LNC surface was slow and gradual and only 33.6 % peptide was desorbed after 6 h (Fig. 3). Moreover, NFL-functionalized LNCs was reported to maintain their characteristics in cell culture medium.²² Therefore, the LNC-fluoNFL2 formulation can be promising for administration by i.v. injection. Additionally, this dialysis experimentation also indicated a high percentage of fluoNFL adsorption in LNC-fluoNFL2. About 94.4 % and 90.4 % of the added peptide were remaining in the dialysis chamber after 30 min and 1 h dialysis respectively for LNC-fluoNFL2, whereas it was only 41.1 % and 9.6 % for the control fluoNFL solution (same initial concentration as LNC-fluoNFL2). Theoretically, up to 58.9 % of the added 3 mM peptide in LNC-fluoNFL2 should be able to cross the dialysis membrane to reach the receiver chamber after 30 min dialysis, if they were free. Therefore, this result also showed that the peptide adsorption percentage in LNC-fluoNFL2 was possibly very high and strongly attached to the LNC surface and further study is necessary to understand the real mechanism.

As the size and zeta potential of the LNCs was altered after fluoNFL adsorption, it could impact the *in vivo* fate of the nanocarrier. Enhanced particle size and positive zeta potential may significantly increase complement protein consumption by nanoparticles, leading to rapid removal from systemic circulation by the mononuclear phagocytic system (MPS).³⁴⁻³⁵ The CH50 unit consumption by the LNC-fluoNFL1 was similar to what shown by the control LNCs (Fig. 4). However, the CH50 unit consumption by the LNC-fluoNFL2 was slightly enhanced

1 compared to the other two formulations. This can be attributed to the increased size as surface
2 area recognition by the complement is proportional to the particle diameter,³⁶ or to the altered
3 zeta potential.³⁵ Overall, the complement consumption by all three formulations was low even at
4 high surface area (calculated by NTA)³⁷ and should not be quickly removed from bloodstream by
5 MPS.

6 Previously, Balzeau *et al.* showed that the internalization of LNC in mouse GBM cells can be
7 enhanced by adsorbing the NFL peptide on its surface.¹⁹ However, cellular uptake on
8 nanocarriers can be cell specific as the interacting plasma membrane composition (*i.e.* ligands,
9 receptors and endocytosis apparatus) vary among cell lines.³⁸ Therefore, as a potential
10 therapeutic strategy for human disease, it was necessary to characterize the internalization
11 kinetics of the LNC with/without the surface-adsorbed NFL peptide in a human GBM cell line at
12 a non-toxic concentration. Moreover, Lépinoux-Chambaud *et al.* reported that the extent and
13 pathway of NFL internalization into U87MG cells were dependent on the extracellular peptide
14 concentration.²³ Therefore, the effect of LNC surface-functionalizing fluoNFL peptide
15 concentration on LNC internalization by U87MG human GBM cells was evaluated in this study.
16 For this purpose, the LNCs were fluorescently labelled by encapsulation of a hydrophobic dye,
17 DiA, and their cellular uptake was quantified by fluorescence-activated cell sorting (FACS). The
18 LNC concentration used for the cellular uptake studies was 1.23 mg/mL, having 11.1 μ M of
19 NFL-peptide in LNC-fluoNFL2. The blank LNCs and the peptide solutions were non-toxic at
20 these concentrations as observed in the MTS assays (Supporting Information: Fig. S1 and S2).
21 To identify and separate dead cells, the FACS samples were suspended in 0.12 % w/v of trypan
22 blue for the measurements in flow cytometer and signals in 655 nm long-pass filter was detected.
23 Trypan blue can enter inside cells with damaged membrane, complex with proteins and emit

fluorescence around 660 nm that can be detected in FACS.³⁹⁻⁴⁰ However, maximum 0.1 % dead cells were detected in the FACS samples which confirms that the LNC concentration used for treatment of cells was non-toxic. The internalization of all three formulations increased with time (Fig. 5). At each time point, the uptake of LNC-DiA-fluoNFL2 was significantly higher compared to LNC-DiA-fluoNFL1 and LNC-DiA, whereas the internalization of LNC-DiA-fluoNFL1 was significantly higher compared to LNC-DiA. It was also observed that the peptide needs to be absorbed onto the LNC surface (by 24 h stirring) for maximizing LNC internalization as the uptake of 'LNC-DiA and fluoNFL immediate mixture' was significantly lower compared to LNC-DiA-fluoNFL2 (Fig. 6). Therefore, the internalization of nanocapsules into U87MG cells is dependent on the concentration of NFL on LNC surface. Confocal microscopy images visually confirmed the much higher cellular uptake of LNC-DiA-fluoNFL2 compared to LNC-DiA (Fig. 7), and showed that majority of the NFL-functionalized LNCs was into the cytoplasm whereas the LNC-DiA was mostly attached to the cell membrane (Fig. 8). It has been shown for the first time that the NFL peptide concentration (as a targeting-ligand) onto nanocarrier surface can have significant impact on the rate and the extent of the nanovector cellular internalization, on human GBM cells. Therefore, this strategy could be used to improve nanocarrier targeting efficiency to other type of cells in which the peptide can efficiently enter *i.e.* brain neural stem cells.^{18, 22, 41}

Previously, Paillard *et al.* reported that the internalization of untargeted LNCs was not preferentially targeted into GBM cells and entered also healthy astrocytes.³⁸ Additionally, surface-functionalization with the fluoNFL peptide significantly enhanced the uptake of LNCs in NHA, compared to control LNCs (LNC-DiA), NFL being a cell penetrating peptide. Therefore, to investigate the targeting capacity of the LNC-fluoNFL2 towards U87MG cells, LNC-DiA and

LNC-DiA-fluoNFL2 were incubated with NHA and their cellular uptake after 1 h and 6 h was measured and compared with their uptake in U87MG cells (Fig. 9). At 1 h, no significant difference was observed between LNC-fluoNFL2 internalization in NHA and U87MG cells. However, the rate of LNC-fluoNFL2 internalization was much faster in the GBM cells and the nanovector entered significantly more in the cancer cells compared to NHA. Therefore, the cellular internalization of LNC-fluoNFL2 was preferentially targeted towards the human GBM cells compared to healthy cells.

To investigate the possible pathway(s) of LNC-fluoNFL2 internalization in U87MG cells, its uptake was followed in different energetic conditions and in presence of various endocytosis pathway inhibitors. The internalization of the nanocarrier was significantly reduced (compared to 37 °C) when incubated at different energetic conditions (4 °C and ATP-depleted condition) (Fig. 10a). In ATP-depleted conditions, no LNC-DiA-fluoNFL2 uptake was showed after 1 h, but an increase to about 25 % of the n.c after 6 h was observed. On the contrary, at 4°C, no LNC-DiA-fluoNFL2 was internalized after 1 h and 6 h of incubation. This result could suggest that in ATP-depleted conditions, after 6h, these nano-objects were able to be internalized via an independent-energy pathway. But the same result was surprisingly not observed at 4°C. Indeed, a higher rigidity and a lower permeability of lipid bilayer have been demonstrated at low temperature and could explain this difference ⁴².

Thus, the LNC-fluoNFL2 uptake in U87MG cell was an energy-dependent active process. Comparable trend was observed in cellular uptake of the fluoNFL alone (Fig. 10b) which was also mentioned in previously reports.¹⁸

The dependency of cellular uptake on energy indicates that the internalization possibly occurs by endocytosis. To further illustrate about the particular internalization pathway(s) involved, the

cells were pretreated with various inhibitors of the chief endocytosis pathways. Treatment with M β CD depletes cholesterol and inhibits both clathrin- and caveolin-mediated endocytosis, DAM prevents macropinocytosis, chlorpromazine blocks clathrin-dependent endocytosis and PMA impedes caveolin-dependent endocytosis.^{38, 43} As previously reported for the NFL peptide²³ (also observed in our experiments, Fig. 11b), the internalization of the LNC-fluoNFL2 was not dependent on one particular endocytosis pathway, rather on several and its uptake was significantly reduced when cells were pretreated with these inhibitors (Fig. 11a). Taken together, the predominant pathways involved in NFL-functionalized LNC internalization were macropinocytosis, clathrin-dependent and caveolin-dependent endocytosis; similar to the free peptide solution. However, the very low uptake of the non-functionalized LNC into U87MG cells up to 6 h was not suitable to be used as control for evaluating its cellular uptake mechanisms. Moreover, the 24 h time point has been tried for determining the possible LNC internalization pathways. But the cells did not survive up to 24 h in presence of the different endocytosis inhibitors and the mechanism of LNC uptake in U87MG cells could not be determined by this method.

A promising ferrocifen-type anticancer drug FcTriOH was encapsulated in the LNCs. FcTriOH belongs to the hydroxypropyl series of the acyclic ferrocifen family, possibly forms an intrinsically electrophilic and cytotoxic metabolite i.e. tetrahydrofuran substituted quinone methides in living cancer cells, and have shown significantly enhanced activity on hormone-independent breast cancer cells compared to the acyclic ferrociphenol.¹² This was the first time this compound was studied *in vivo* on a human GBM tumor model in mice. The *in vitro* antiproliferative activity of FcTriOH was assessed by MTS assay. The cells were treated with 0.1-100 μ M of FcTriOH and its formulations for 72 h. Up to 0.1 μ M, cell survival was above 80

% for all treatment groups (Fig. 12). Between 0.1-10 μ M, the cell survival percentage drastically reduced for cells treated with FcTriOH, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2 resulting in IC₅₀ values of 1.31 μ M, 1.05 μ M and 0.46 μ M respectively. The survival of the cells treated with control LNC reduced significantly between 10 and 100 μ M with an IC₅₀ of 22.5 μ M. Corresponding concentrations of fluoNFL solution did not alter cell viability which was also reported previously.¹⁸

In the preliminary *in vivo* study, an U87MG subcutaneous GBM tumor model was used to evaluate potential tumor reduction efficacy or possible toxicity after two tail vein injections equivalent to 20 mg/kg FcTriOH. As no previous reports about this FcTriOH administration in animals were available, the dose was chosen based on previous *in vivo* studies involving other ferrocifen molecules.⁴⁴ The two i.v. injections were given on day 7 and day 10. A tendency of relative tumor volume gradual reduction was observed since the beginning of the treatment with FcTriOH-loaded LNCs. A significant difference *i.e.* 40.1 % and 44.2 % lower relative tumor volume for LNC-FcTriOH and FcTriOH-fluoNFL2 respectively, compared to the saline treated group, was observed on by day 17 (Fig. 13) which was maintained up to day 22. The mouse showed no behavioral signs of pain or irritation immediately after the injection. Additionally, the weight of the mice never reduced and signs of pain and distress were not observed throughout the study. However, the tumor reduction effect of the FcTriOH treatments was not observed from day 24 (two weeks after the last injection). The tumor rapidly grew back and no significant difference in relative tumor volume was observed. This possibly occurs as the drug is eliminated resulting its antiproliferative effect to fade and the tumor to grow back. In fact, several preclinical studies have used much higher number (6 to 20) of i.v. injections⁴⁴⁻⁴⁵ and observed a significant difference on tumor growth. In clinical practice, chemotherapy is generally

administered in several cycles; a treatment period followed by a waiting period for the patient to wash-out and recover from the side effect of the drug. The cycle frequencies are optimized depending on the treatment used. In future studies, the number of injections and/or dose should be increased to possibly achieve tumor regression after FcTriOH-loaded LNC treatment. Although the relative tumor volume of LNC-FcTriOH treated and LNC-FcTriOH-fluoNFL2 treated groups on day 17 was significantly lower from saline treated groups, the difference among themselves were not significant. However, the average value of relative tumor growth was slightly lower for NFL-functionalized LNC treated groups. The tendency may be more clearly observed if more injections are given in the future studies.

The NFL-peptide concentration of LNC surface can be further increased for additional enhancement of its internalization in human GBM cells. However, the currently used preparation technique is not suitable for this purpose as precipitates were observed in fluoNFL peptide solutions above 3 mM, probably due to its aqueous solubility limit. Additionally, covalent coupling of NFL at the distal end of long-chain PEG has been described to hamper its GBM-targeting properties.¹⁹ However, addition of NFL solution/suspension above 3 mM in LNC dispersions resulted a viscous and hydrogel like formulation (data not shown), which could be interesting for local application in the cavity after surgical removal of the tumor.⁴⁶

EXPERIMENTAL

Materials

Macrogol 15 hydroxystearate (Kolliphor® HS15) was purchased from BASF (Germany). Hydrogenated phosphatidylcholine from soybean (Lipoid S PC-3) was provided from Lipoid GmbH (Germany), caprylic/capric triglycerides (Labrafac Lipophile WL1349) was supplied by

Gattefosse (France). FcTriOH was provided by PSL Chimie ParisTech (France), Feroscan SME. 5,6-Carboxyfluorescein labelled NFL.TBS-40.63 peptide (fluoNFL) was purchased from Polypeptide Laboratories (France).

The human glioblastoma cell line U87MG was collected from ATCC (USA). Normal human astrocytes (NHA), astrocyte basal medium (ABM), SingleQuots™ kit supplements & growth factors, L-glutamine, penicillin-streptomycin and Dulbecco's modified Eagle's medium with 1 g/L L-glucose (DMEM) were provided by Lonza (France). Methyl- β -cyclodextrin (M β CD), 5-(N,N-dimethyl) amiloride hydrochloride (DAM), chlorpromazine (CP), phalloidin-tetramethylrhodamine-B-isothiocyanate (phalloidin-TRITC), sodium azide and 2-deoxy-D-glucose were purchased from Sigma (Germany). Phorbol-12-myristate-13-acetate (PMA) was collected from Abcam (France). 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA), 4',6-diamidino-2-phenylindole (DAPI), Trypsin-EDTA 1x, non-essential amino acids solution 100x (NEAA), fetal bovine serum (FBS) and ProLong Gold antifade were collected from Thermo Fisher Scientific (USA). 3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) was purchased from Promega (USA).

Normal human serum (NHS) was provided by the "Etablissement Français du Sang" (Angers, France). Sheep erythrocytes and hemolysin were purchased from Eurobio (France). Sodium chloride (NaCl) was purchased from Prolabo (Fontenay-sous-bois, France). Ultra-pure water (UPW) was obtained from a Millipore filtration system. All the other reagents and chemicals were of analytical grade.

Preparation of lipid nanocapsules

Preparation of stock lipid nanocapsules

Stock LNC (LNC-stock) was prepared using phase inversion temperature technique.²¹ In brief, Kolliphor® HS15 (16.9 % w/w), Lipoid® S PC-3 (1.5 % w/w), Labrafac Lipophile WL1349 (20.6 % w/w), NaCl (1.8 % w/w) and UPW (59.2 % w/w) were mixed under magnetic stirring at 60 °C for 15 min. Three heating-cooling cycles were performed between 90 °C and 60 °C. During the last cooling step, when the temperature was in the phase inversion zone (78-83 °C), ice-cold UPW was added (final concentration 88.4 % w/w) to induce irreversible shock and form the LNC-stock. The nanocapsules were then passed through 0.2 µm cellulose acetate filter to remove any aggregates and stored at 4 °C.

DiA-labelled stock LNC was prepared by incorporating 0.1% (w/w) DiA in the formulation at the first step with other excipients.

Preparation of fluoNFL functionalized lipid nanocapsules and lipid nanocapsules

1 mL of stock LNC (LNC-stock) was stirred at room temperature for 24 h with 0.369 mL of 1 mM (0.86 % w/w) (similar to ^{19, 22}) or 3 mM (2.57 % w/w) fluoNFL solution (in water) to prepare the fluoNFL functionalized LNCs (LNC-fluoNFL1 and LNC-fluoNFL2 respectively). Similarly, 1 mL of the LNC stock was stirred at room temperature for 24 h with 0.369 mL of UPW to produce final control LNCs.

The DiA (0.1 % w/w) labelled LNCs was also functionalized with the fluoNFL by same method as LNC-fluoNFL1 and LNC-fluoNFL2.

Preparation of FcTriOH-loaded lipid nanocapsules

The FcTriOH-loaded LNC (LNC-FcTriOH) was prepared similarly as control LNCs, except FcTriOH (0.9 % w/w) was added at the first step of the formulation with the other excipients.

Subsequently, fluoNFL was adsorbed at their surface using identical method as LNC-fluoNFL2 to produce drug loaded NFL-functionalized LNCs.

Optimization of lipid nanocapsules for in vivo studies

For *in vivo* studies, the amount of ice-cold UPW used to induce shock to produce the LNCs was adjusted (final concentration 70.9% w/w) to produce concentrated LNCs according to previously published article.²⁴ NaCl concentration was also adjusted to keep the final formulations isotonic with blood.

Characterization of the lipid nanocapsules

Dynamic light scattering, laser-Doppler electrophoresis and nanoparticle tracking analysis

The mean diameter and polydispersity index (PDI) of the LNCs were determined by dynamic light scattering (DLS) technique using Zetasizer Nano ZS (Malvern Instruments Ltd, UK). The LNCs were diluted 100-folds in UPW before the analysis. The measurements were performed at backscatter angle of 173°. The measured average values were calculated from 3 runs, with 10 measurements within each run.

Zeta potential of the nanocarriers was measured using laser Doppler micro-electrophoresis using Zetasizer Nano ZS (Malvern Instruments Ltd, UK).

Additionally, the particle concentration in the control LNC dispersion was determined using nanoparticle tracking analysis (NTA) as described previously.³⁷ The NTA was carried out using the NanoSight NS300 (Malvern Instruments Ltd, UK). Briefly, the NDDS samples were diluted to optimum concentrations with UPW and were infused in the sample chamber using a syringe pump at 30 µL/min rate. A 405 nm laser was used to illuminate the particles, and their Brownian

1 motion was recorded into three 60 s videos (25 fps) using the sCMOS type camera of the
2 instrument. Subsequently, the NTA software (NTA 3.2 Dev Build 3.2.16) analyzed the
3 recordings, tracked the motion of the particles and calculated the number of particles in the
4 samples. The experiment was performed in triplicate.

6 ***High-performance liquid chromatography***

7 The peptide concentration was indirectly measured by quantifying the free peptide present in
8 the formulations using a supplier recommended HPLC method. Briefly, the fluoNFL
9 functionalized LNCs were filtered by centrifugation at 4000 g for 30 min using Amicon Ultra-
10 0.5 mL centrifugal filters having molecular weight cut off (MWCO) 100 kD (Millipore). The
11 filtrate containing the free fluoNFL was collected and the peptide dosage was performed in a
12 HPLC system (Waters, France). A C18 analytical column (250 x 4.6 mm, 5 μ m, Waters, France)
13 was used at room temperature. 0.1% TFA in UPW and 0.1% TFA in acetonitrile were used as
14 mobile phases (gradient: 80:20 \rightarrow 55:25, 25 min). Flow rate was 1 mL/min, injection volume
15 was 10 μ L and fluoNFL was quantified by an UV detector at λ of 220 nm. Analysis of the data
16 was performed by Empower 3 software (Waters). Retention time was of 18 min. Calibration
17 curves were established by quantifying the area under the curves (AUCs) of 1-100 μ g/mL
18 solutions of fluoNFL in UPW. The peptide solution and LNCs alone were also filtered and
19 quantified as positive and negative controls.

20 To quantify total (encapsulated and unencapsulated) drug concentration, LNCs were broken by
21 mixing vigorously with an appropriate volume of ethanol (40 folds for LNCs prepared for *in*
22 *vitro* experiments, 100-folds for concentrated LNCs prepared for *in vivo*) to keep dissolved drug
23 concentration between 5-75 μ g/mL. To quantify unencapsulated drug concentration,

formulations were placed on centrifugal concentrator devices with polyethersulfone membrane (MWCO 30 kD, Amicon Ultra-500, Millipore) and centrifuged at 4000 g for 30 minutes to separate the free drug from the rest of the formulation. The filtrates containing unencapsulated drug were collected and ethanol (2-folds) was added to solubilize any undissolved drug. Drug dosage in the above-mentioned samples was performed in a HPLC system (Waters, France). A C18 analytical column (250 x 4.6 mm, 5 µm, Waters, France) was used at room temperature. UPW and acetonitrile (45:55, v/v) were used as mobile phases. Flow rate was 1 mL/min, injection volume was 10 µL and FcTriOH was detected at 304 nm. Analysis of the data was performed by Empower 3 software (Waters). Retention time of FcTriOH was 8.1 min.

Encapsulation efficiency, EE (%) was calculated using the following equation:

$$EE (\%) = \frac{(\text{Total drug conc. in LNC} - \text{unencapsulated drug conc. in LNC}) \times 100}{\text{Initial drug conc. in LNC}}$$

Drug loading was calculated using the following equation:

$$\text{Drug loading (\% w/w)} = \frac{\text{Mass of encapsulated drug in 1 mL LNC dispersion} \times 100}{\text{Total mass of excipients in 1 mL LNC dispersion}}$$

Interaction between LNCs and fluoNFL

The LNC-fluoNFL2 and control LNCs were diluted in UPW or in various concentrations (0.005, 0.05, 0.15, 0.25, 0.5 and 1 M) of NaCl or Tris buffer, incubated for 30 minutes before measuring their size by DLS.²²

Additionally, LNC-fluoNFL2 and control fluoNFL solutions were taken in dialysis bags (MWCO 100 kD, Spectra/Por[®] biotech grade cellulose ester membrane, SpectrumLabs, Netherlands) and dialyzed against Tris buffer (0.05 M, pH 7.4) at 37°C, stirred at 75 rpm. At various time points (0.25, 0.5, 0.75, 1, 2, 3, 4, 6 and 24 h) samples were collected from the

receiver chamber and amount of the free peptide was quantified using the HPLC method mentioned above.

Complement consumption assay (CH50 assay)

The residual hemolytic capacity of NHS towards antibody-sensitized sheep erythrocytes after incubation with different LNC formulations was measured to evaluate the complement activation by the formulations.⁴⁷ In brief,³⁷ aliquots of NHS were incubated with increasing concentrations of the LNCs at 37 °C for 1 h. Subsequently, the different volumes of the NHS were incubated with a fixed volume of hemolysin-sensitized sheep erythrocytes at 37 °C for 45 min. The volume of serum that can lyse 50 % of the erythrocytes was calculated (“CH50 units”) for each sample and percentage of CH50 unit consumption relative to negative control was determined as described previously.³⁴ Particle number in the LNC dispersion was determined by NTA and nanocarrier concentration per mL of NHS was calculated according to following equation:

$$\text{Particle number per mL of NHS} = \text{Particle conc. in NDDS dispersion} \times \frac{\text{vol. of NDDS added}}{\text{vol. of NHS}}$$

Subsequently, surface area of the NDDSs per mL of NHS was calculated according to the following equation:

$$\text{Surface area} = \text{Particle number per mL of NHS} \times \pi \times (\text{average particle diameter})^2$$

The CH50 unit consumption by the different LNCs was compared by plotting the percentage of CH50 unit consumption as a function of their surface area.

Cell culture

The human glioblastoma cell line U87MG was cultured at 37 °C under 5 % CO₂ in DMEM supplemented with 10 % FBS, 5% L-glutamine, 5 % NEAA and 5 % penicillin-streptomycin.

NHA was cultured at 37 °C under 5 % CO₂ in ABM supplemented by the 'AGM SingleQuot™ Kit'. The cells were passaged once they were about 70 % confluence.

Flow cytometry

Kinetics of LNC internalization in U87MG cells

The kinetics of internalization of the DiA-labelled LNCs (LNC-DiA, LNC-DiA-fluoNFL1 and LNC-DiA-fluoNFL2) in U87MG cells was assessed using the BD FACSCanto™ II flow cytometer (BD Biosciences). In brief, cells were seeded in 6-well plates at 5×10^5 cells/well concentration for 24 hours. Subsequently, they were treated with the different DiA-labelled LNCs (1.23 mg/mL) for 0.5, 1, 6 and 24 h. Afterwards, the cells were washed three times with ice-cold phosphate buffer saline 1x (PBS), detached by incubating 5-10 minutes with Trypsin-EDTA 1x. The cells were then centrifuged at 2000 rpm for 5 minutes, the supernatant was aspirated and the cell pellet was re-dispersed in PBS. The centrifugation and re-dispersion cycle was repeated twice more. Finally, the cells were suspended in trypan blue (final trypan blue concentration 0.12% w/v) and the percentage of DiA positive (DiA^{+ve}) or FAM positive (FAM^{+ve}) cells were analyzed by the flow cytometer. Each experiment was performed in triplicate and 20,000 events per sample were analyzed in each experiment.

Targeting-capability of fluoNFL-functionalized LNCs towards GBM cells compared to healthy cells

To assess the targeting-capability of the fluoNFL-functionalized LNC towards GBM cells compared to healthy cells, NHA was treated for 1 h and 6 h with LNC-DiA-fluoNFL2 (method

mentioned above) at 37 °C and percentage of DiA^{+ve} cells was measured using the above-mentioned method, and compared with the results of U87MG cells.

Mechanism of fluoNFL-functionalized LNCs internalization in U87MG cells

To evaluate the dependency of NFL-functionalized LNC cellular internalization on energy, U87MG cells were pre-incubated for 30 min at 4 °C or pretreated for 30 minutes at 37 °C (NaN₃ 10 mM and 2-deoxy-D-glucose 6 mM) to deplete cellular ATP²³. Subsequently, the cells were treated for 1 and 6 h with the LNC-DiA-fluoNFL2 and percentage of DiA^{+ve} cells were measured by the above-mentioned method.

To investigate the possible pathways of LNC-DiA-fluoNFL2 internalization in U87MG, cells were pretreated with different inhibitors (MβCD 10 mg/mL, DAM 1 mM, CP 50 μM and PMA 10 μg/mL) for 30 min at 37 °C²³ followed by 1 h treatment with the nanocarrier and percentage of DiA^{+ve} was quantified.

In all the above-mentioned conditions (37 °C, pre-incubation at 4 °C, pre-treatment for ATP depletion, and pre-treatment with various inhibitors), internalization of fluoNFL (at equivalent concentration of LNC-DiA-fluoNFL2) in U87MG cells was assessed by measuring FAM^{+ve} cells to assess if the fluoNFL by itself regulates the internalization of NFL-functionalized LNC.

Confocal microscopy

To visualize the potential effects of the fluoNFL peptide on LNC internalization, U87MG cells were seeded (3×10^4 cells/well) in 24 well plates containing coverslips and incubated at 37 °C for 72 h (medium was carefully replaced every 24 h) to allow the cells to grow on the coverslips. Subsequently, the cells were treated with 1.23 mg/mL of LNC-DiA or LNC-DiA-fluoNFL2 for 1

h and 6 h at 37 °C. Afterwards, the cells were washed three times with PBS and fixed with 4 % paraformaldehyde for 20 min at room temperature. Then, the cells were washed twice with PBS and permeabilized by incubation with 0.1 % Triton X-100 for 10 min. The cells were washed twice with PBS and incubated with 0.7 μ M of phalloidin-TRITC for 1 h at room temperature. Subsequently, the cells were washed twice with PBS and incubated with 3 μ M DAPI for 10 minutes. Finally, the cells were washed 3-times with PBS and the coverslips were mounted using ProLong Gold antifade mounting medium. The cells were then visualized and images were captured by a confocal microscope (LSM 700 Zeiss). DAPI was excited with a 405 nm wavelength and recorded at 409-453 nm (blue channel), DiA and fluoNFL were excited with a 458 nm wavelength and recorded at 558-666 nm (green channel) whereas TRITC was excited with a 561 nm wavelength and recorded at 564-632 nm (red channel).

In vitro efficacy

Viability of the U87MG cells to various LNC treatments was assessed by MTS assay.¹⁹ In brief, the U87MG cells were seeded in 96 well plates (5000 cells/well) and incubated for 24 h. Then the medium was replaced with various concentrations of LNCs (60-600,000 folds diluted in DMEM) (LNC-blank, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2), FcTriOH (22-220,000 folds dilution in DMEM) and fluoNFL (125-4,983,383 folds dilution in DMEM) and treated for 72 hours at 37 °C. After that, each well content was replaced with 100 μ L of fresh DMEM. Additionally, 20 μ L of MTS-PMS (20:1) mixture was added in each well and incubated at 37 °C for 2 h. Absorbance of the samples at 490 nm was recorded using a microplate reader (SpectraMax M2, Molecular Devices). The absorbance of the cells incubated with only DMEM

was considered as 100 % of cell survival (Abs^{+ve}), and the cells treated by 0.5 % Triton X-100 was considered as 0 % (Abs^{-ve}). Cell survival was calculated using the following equation:

$$\text{Cell survival (\%)} = [(Abs^{\text{sample}} - Abs^{-ve}) \div (Abs^{+ve} - Abs^{-ve})] \times 100$$

Preliminary in vivo study in ectopic xenograft model

The preliminary *in vivo* study was performed following the guidelines of the European regulations. The experimental protocol was approved by the ‘French Ministry of National Education, Higher Education and Research’: APAFIS#8293-2017032217458665v3. Seven weeks old female NMRI nude mice were collected from Janvier Labs (France). The animals were kept in the animal facility for one week for acclimatization and were given sufficient food and water throughout the study.

After acclimatization period, the animals were anesthetized by temporary exposure to 2 % isoflurane in oxygen to induce anesthesia followed by 1.5 % isoflurane in oxygen delivered by face mask to maintain it. The U87MG cells were trypsinized and washed three times before injected subcutaneously in the right flank of the mice (2×10^6 cells in 50 μ L PBS). When the tumor became palpable, tumor volume was measured using an electronic caliper using the following equation: $\text{Volume} = \pi/6 \times \text{length} \times \text{width}^2$. Seven days after cell injection, the mice were divided into 5 groups to have similar average tumor volume. The animals were anesthetized (by above mentioned method) and received the following treatments by injections in the tail vein on day 7 and day 10- Group 1: 70 μ L Saline (n = 7); Group 2: 70 μ L of LNC-blank equivalent to 822.4 mg LNC per kg of body weight (n = 8); Group 3: 70 μ L of LNC-blank-fluoNFL2 equivalent to 822.4 mg LNC and 21.5 mg peptide per kg of body weight (n = 8), Group 4: 70 μ L of LNC-FcTriOH equivalent to 20 mg FcTriOH per kg of body weight (equivalent to 822.4 mg

LNC per kg of body weight) (n = 8); Group 5: 70 μ L of LNC-FcTriOH-fluoNFL2 equivalent to 20 mg FcTriOH per kg of body weight (equivalent to 822.4 mg LNC and 21.5 mg peptide per kg of body weight) (n = 8). The length and width of the tumor was followed regularly (every day in the first week of treatment and then 3-times a week). Weight and behavior of the animals were daily followed.

Statistical analysis

The experiments were performed at least 3 times. Results obtained from the experiments were analyzed statistically using GraphPad Prism[®] software. Mean and standard deviation (SD) were determined and values are represented as Mean \pm SD. T-test or One way analysis of variance (ANOVA) (with Tukey post-test to compare among individual groups, and Dunnett's post-test to compare with control) was performed in the respective fields. P-value less than 0.05 (p <0.05) was considered to be statistically significant.

CONCLUSIONS

In this study, we have shown that surface-functionalization with NFL peptide can enhance the uptake of LNCs in human GBM cells in a dose-dependent manner. Moreover, the peptide-functionalized LNCs reached the cytoplasm at much higher concentration compared to the non-functionalized control LNCs. Additionally, the peptide functionalized LNCs were preferentially internalized into GBM cells compared to healthy human astrocytes showing the targeting capacity of the nanovector. The internalization of this nanocarrier in the U87MG cells was energy-dependent and occurred by a combination of macropinocytosis, clathrin-mediated and caveolin-mediated endocytosis, similar pathway as the NFL peptide solution. Encapsulation for

the first time of this FcTriOH in the GBM targeting LNCs resulted in a decreased IC₅₀. The preliminary *in vivo* study in an ectopic human GBM xenograft model showed that the drug-loaded LNC therapy did not cause any pain or distress after i.v. administration and their tumor reduction efficacy was promising. However, more cycles of chemotherapy seemed necessary in future experiments to avail the benefit of functionalization. Moreover, further experiments on an orthotopic xenograft mouse model, after i.v. injections, will be necessary to consider the main biological barriers to be crossed to reach the target: BBB and BBTB⁴⁸. Overall, enhancement of NFL peptide concentration on LNC surface is a promising strategy for greater and targeted nanocarrier internalization into human glioblastoma cells, and the FcTriOH-loaded LNCs are promising therapy approach for glioblastoma.

CONFLICTS OF INTEREST

There are no conflicts to declare.

ACKNOWLEDGEMENTS

The authors would like to thank P. Legras (Service Commun d'Animalerie Hospitalo-Universitaire, Angers, France) for his technical assistance in animal experiments, C. Guillet (Plate-forme d'Analyse Cellulaire et Moléculaire, University of Angers) for her procedural guidance in the FACS analyses, R. Perrot (Service Commun d'Imageries et d'Analyses Microscopiques, University of Angers) for his support in confocal microscopy and the SME Feroscan for its supply in ferrocifens. This work was funded by the NanoFar Consortium of the Erasmus Mundus program and “fonds Léon Fredericq”, CHU, University of Liege, Liege, Belgium. The authors also thank ‘La ligue contre le cancer 49’ for their support in this work.

Supporting Information

Electronic Supplementary Information (ESI) available: Figures showing the results of MTS assays of blank LNCs and fluoNFL solutions. See DOI: 10.1039/x0xx00000x

List of abbreviations

BBB: blood-brain barrier; BBTB: blood-brain tumor barrier; CP: chlorpromazine; DAM: 5-(N,N-dimethyl) amiloride hydrochloride; DiA: 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide); DLS: dynamic light scattering; EE: Encapsulation efficiency; EPR: enhanced permeability and retention; FcTriOH: 4-ferrocenyl-5,5-bis(4-hydroxyphenyl)-pent-4-en-1-ol; fluoNFL: FAM-labelled NFL-TBS.40-63 peptide; GBM: glioblastoma; HPLC: high performance liquid chromatography; IC₅₀: inhibitory concentration 50; LNC: lipid nanocapsule; M β CD: Methyl- β -cyclodextrin; NDDS: nanosized-drug delivery system; NFL: neurofilament light subunit derived 24 amino acid tubulin binding site peptide NFL-TBS.40-63; NHA: normal human astrocytes; NHS: normal human serum; NTA, nanoparticle tracking analysis; PDI: polydispersity index; phalloidin-TRITC: phalloidin-tetramethylrhodamine-B-isothiocyanate; PMA: phorbol-12-myristate-13- acetate; UPW: ultra-pure water.

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Supporting Information

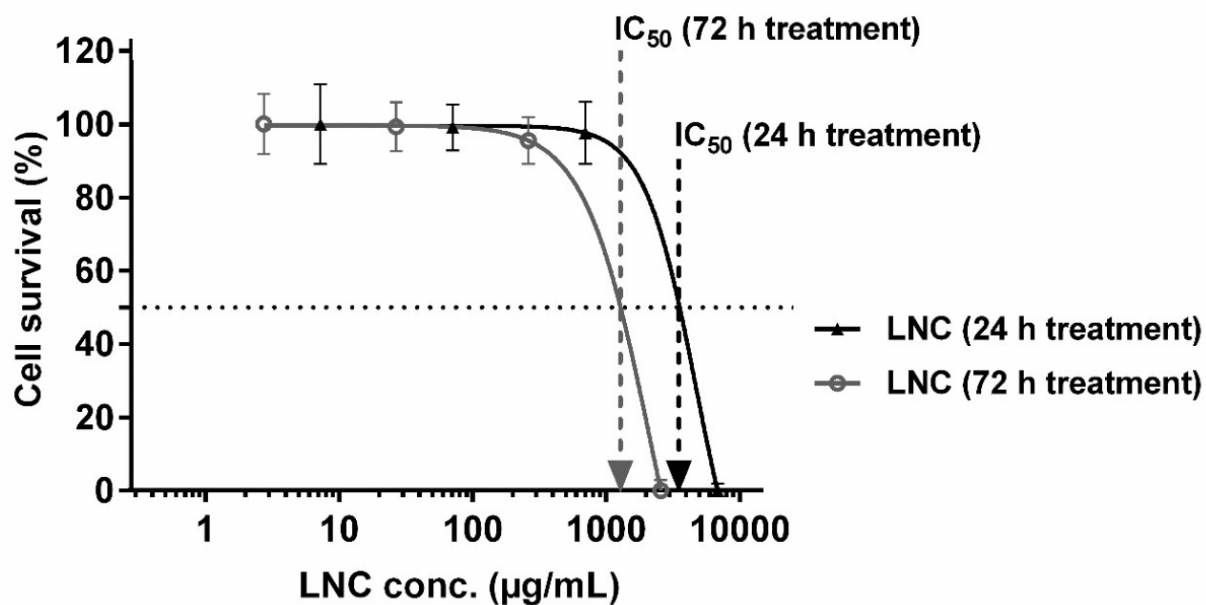


Figure S1. Survival percentages of U87MG cells after 24 h and 72 h treatment with various concentrations of blank LNCs.

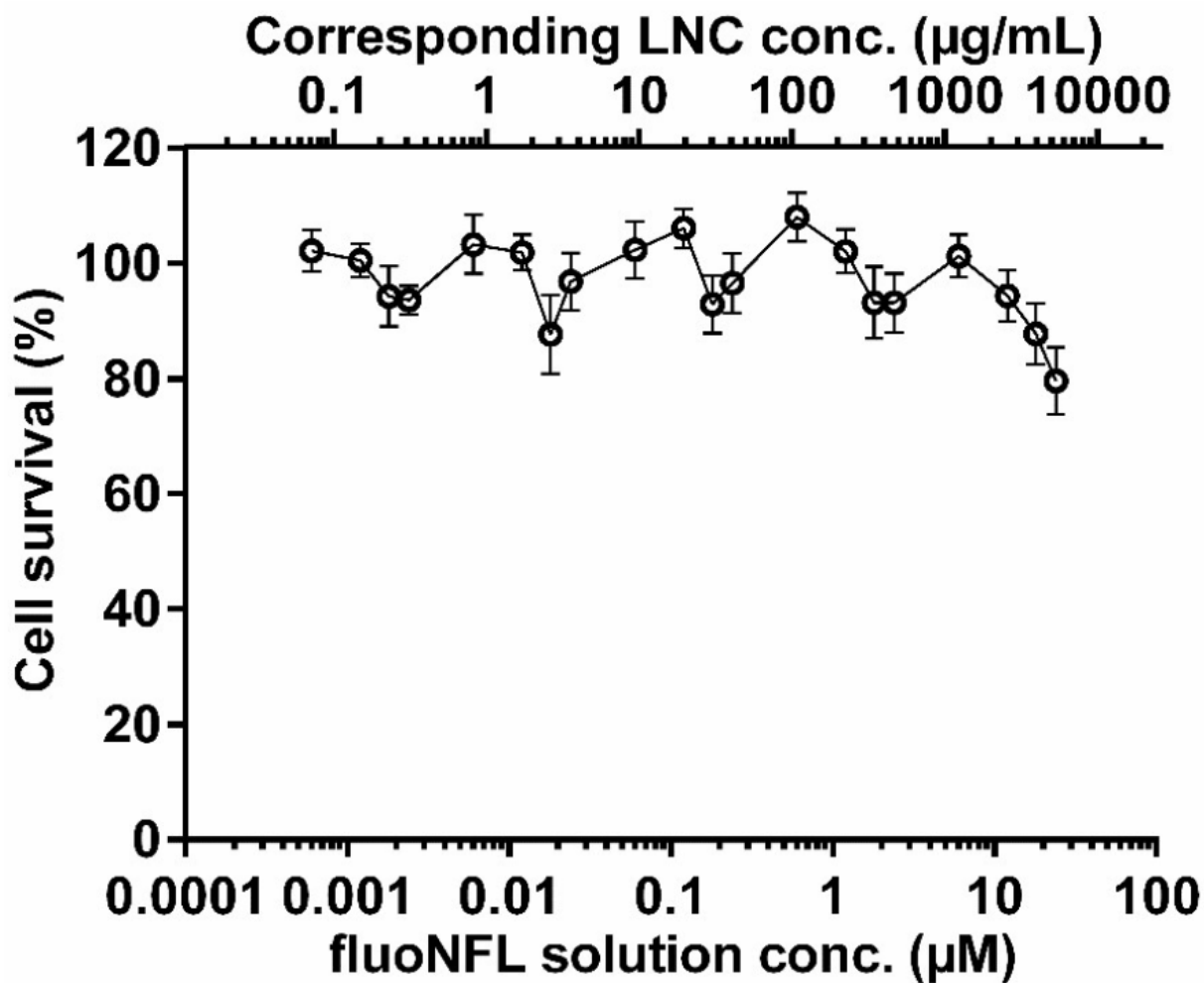


Figure S2. Survival percentages of U87MG cells after 72 h treatment with various concentrations fluoNFL solution.

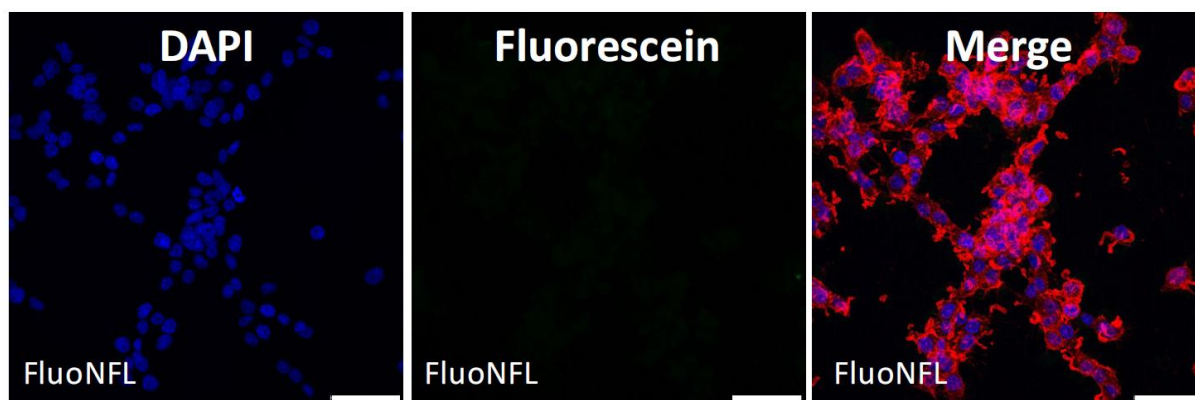


Figure S3. Representative confocal microscopy images of fluoNFL internalization into U87MG. Cells were treated at 37 °C for 6 h with 1 μM of fluoNFL. Blue is DAPI staining (nuclei), green is carboxyfluorescein (fluoNFL) and red is phalloidin-TRITC staining (F-actin, cytoskeleton). White bar = 50 μm.

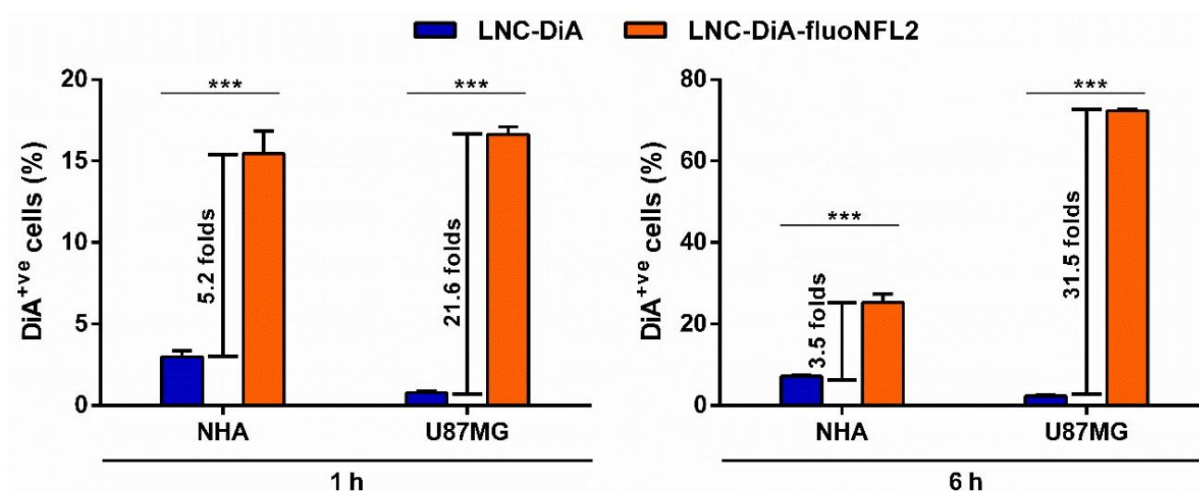


Figure S4: Enhanced LNC Internalization into NHA and U87MG cells due to LNC surface functionalization using fluoNFL peptide. The cells were incubated with 1.23 mg/mL of LNCDiA and LNC-DiA-fluoNFL2 for 1 h and 6 h. Twenty thousand events per sample were analyzed and percentages of DiA^{+ve} cells were measured. The experiments were performed in triplicate. Statistical analysis was performed with t-test ($p < 0.05$ is denoted by (*), $p < 0.01$ by (**), and $p < 0.001$ by (***), $n=3$).