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

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ENHANCED AND PREFERENTIAL INTERNALIZATION OF LIPID NANOCAPSULES INTO HUMAN GLIOBLASTOMA CELLS: EFFECT OF SURFACE-FUNCTIONALIZING NFL PEPTIDE

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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.

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

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In vitro evaluation on human GBM cells

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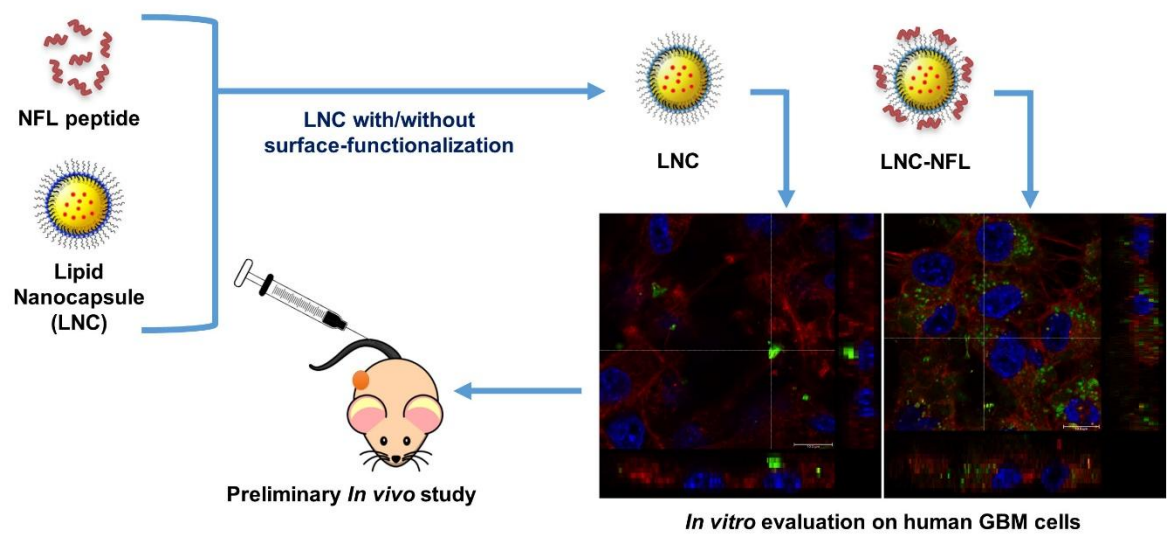
8 Surface functionalized LNC showed a preferential internalization into GBM cells and
9 demonstrated a significant decrease of the tumor volume.
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2 **INTRODUCTION**

3   Glioblastoma multiforme (GBM) is one of the most prevalent, and fatal primary brain tumors

4   classified as the World Health Organization as a grade IV CNS tumor.[1] Although remarkable

5   progress in diagnostic methods and treatment strategies has been achieved in the last few

6   decades, the median survival only altered from 8.3 to 14.6 months over the last 60 years after

7   present multimodal therapy (surgical resection followed by radiotherapy plus chemotherapy).[2-4]

8   Therefore, new therapeutic approaches for treatment of GBM are necessary.

9   Nanosized-drug delivery systems (NDDSs) have appeared as a promising strategy for drug

10   delivery against cancer, including brain cancers. The NDDSs can have numerous beneficial

11   characteristics *i.e.* prolonged blood circulation time, improved bioavailability of hydrophobic

12   drugs, controlled drug release and site-targeted drug delivery.[5] Moreover, long circulating

13   nanocarriers with appropriate size may accumulate in malignant brain tumors after crossing the

14   integrity impaired blood-brain barrier (BBB) by enhanced permeability and retention (EPR)

15   effect, and improve survival time of animals.[6] Among various nanocarriers, lipid nanocapsules

16   (LNCs) have been reported in numerous literatures as promising NDDS for carrying

17   hydrophobic drugs due to their characteristic oily core.[7] One of the promising features of LNC

18   formulation is its easy and organic solvent free preparation technique that can be easy to scale-up

19   for future industrial purpose.[8] LNCs were evaluated and showed promising *in vitro* and *in vivo*

20   results against GBM in numerous studies.[9-11] A novel ferrocifen-type anticancer molecule, 4-

21   ferrocenyl-5,5-bis(4-hydroxyphenyl)-pent-4-en-1-ol (FcTriOH) (Fig. 1), was encapsulated in the

22   LNCs. The FcTriOH belongs to the newer hydroxypropyl series of ferrocifens which can convert

23   into novel tetrahydrofuran-substituted quinone methide.[12-13] Compared to the corresponding

24   acyclic series ferrocifen, FcTriOH showed about 6-folds lower IC_{50} (0.11 ± 0.02 μM) against

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

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

1   of the functionalized-LNC into the human GBM cell line was assessed. Finally, a preliminary *in*

2   *vivo* study was performed on an ectopic human GBM tumor model in mice in order to observe

3   possible therapeutic effects after systemic delivery of the formulations.

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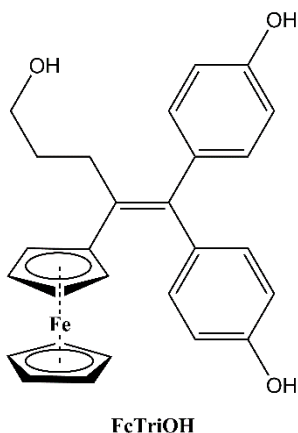
5   **Figure 1:** Structure of FcTriOH: 4-ferrocenyl-5,5-bis(4-hydroxyphenyl)-pent-4-en-1-ol.

6   **RESULTS**

7   **Physicochemical characteristics of the nanocapsules**

8   LNCs were prepared by phase-inversion technique as previously described.[21] The surface of

9   the LNCs was functionalized by adding 0.86 and 2.57 % (w/w) fluoNFL peptide in the LNC

10   dispersion to prepare LNC-fluoNFL1 and LNC-fluoNFL2 respectively. The functionalization

11   occurred by adsorption.[19, 22] The initial molar concentration of NFL peptide for the LNC-

12   fluoNFL1 was kept same as previously published literatures (1 mM)[19, 22] whereas it was 3-folds

13   higher in LNC-fluoNFL2. Final concentrations of the adsorbed fluoNFL (% w/w), determined by

14   HPLC, on LNC-fluoNFL1 and LNC-fluoNFL2 was of 0.40 ± 0.01 % and 2.49 ± 0.01 %

15   respectively.

16   Particle size, PDI and zeta potential of the different nanocapsule formulations determined by

17   Dynamic Light Scattering (DLS) and laser Doppler electrophoresis are given in Table 1. The

18   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 ± 1**	0.12 ± 0.02**	0.5 ± 0.7**
LNC-fluoNFL2	64 ± 1***	0.15 ± 0.02***	4.9 ± 1.5***
LNC-FcTriOH	50 ± 2***	0.06 ± 0.02	-2.3 ± 1.3
LNC-FcTriOH-fluoNFL2	58 ± 1	0.15 ± 0.08*	3.4 ± 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

1   The interaction between the LNC surface and NFL peptide in a formulation equivalent to

2   LNC-fluoNFL1 was described previously.[22] To understand the interaction between the LNC

3   surface and fluoNFL, the LNC-fluoNFL2 and LNC as control were incubated for 30 min in

4   UPW and different concentrations of NaCl or Tris buffer pH 7.4. Subsequently, their size was

5   measured in DLS (Fig. 2). The size of LNC-fluoNFL2 remained significantly different compared

6   to control LNCs as NaCl concentration was increased up to 1 mM. However, as concentration of

7   Tris buffer increased above 0.05 M, the size of LNC-fluoNFL2 was reduced and its significant

8   difference compared to control LNCs was lost.

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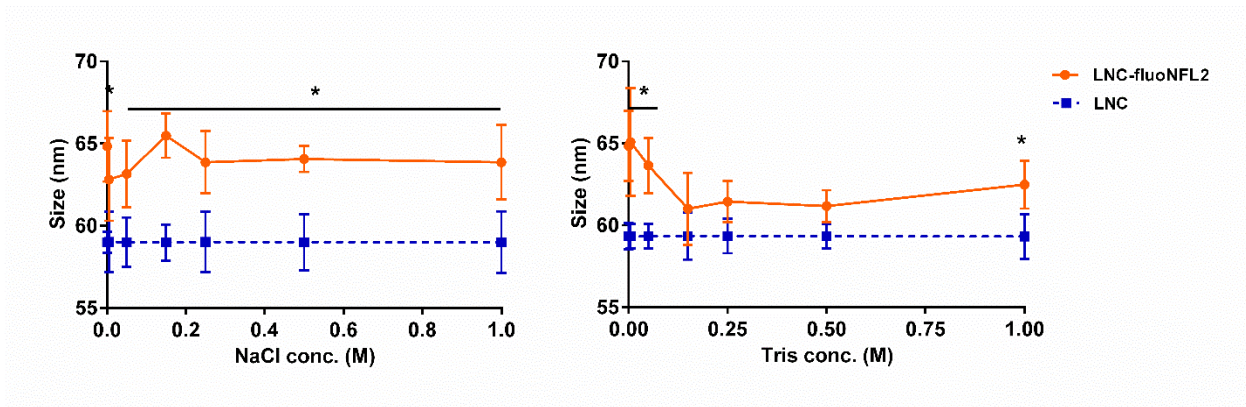
11 **Figure 2:** Mean particle sizes of control LNCs and LNC-fluoNFL2 in various concentrations of

12 NaCl and Tris buffer (t-test. $p < 0.05$ is denoted by (*), $p < 0.01$ by (**) and $p < 0.001$ by (***), n

13 = 3).

14 Moreover, LNC-fluoNFL2 was dialyzed against 0.05 M Tris buffer at 37 °C and 75 rpm using

15 a dialysis bag having MWCO 100 kD. At various time points, the amount of fluoNFL in the

16 receiver chamber (desorbed from the formulation) was quantified by HPLC. As control, the

17 peptide solution was dialyzed and quantified in the receiver chamber. The control peptide

18 solution reached the receiver chamber very quickly and more than 90 % of the peptide was

1   recovered by 1 h. However, a slow and gradual desorption of the peptide was observed (Fig. 3)

2   from the LNC surface and only 6 % peptide desorption occurred by 30 min and reached 33 % by

3   6 h.

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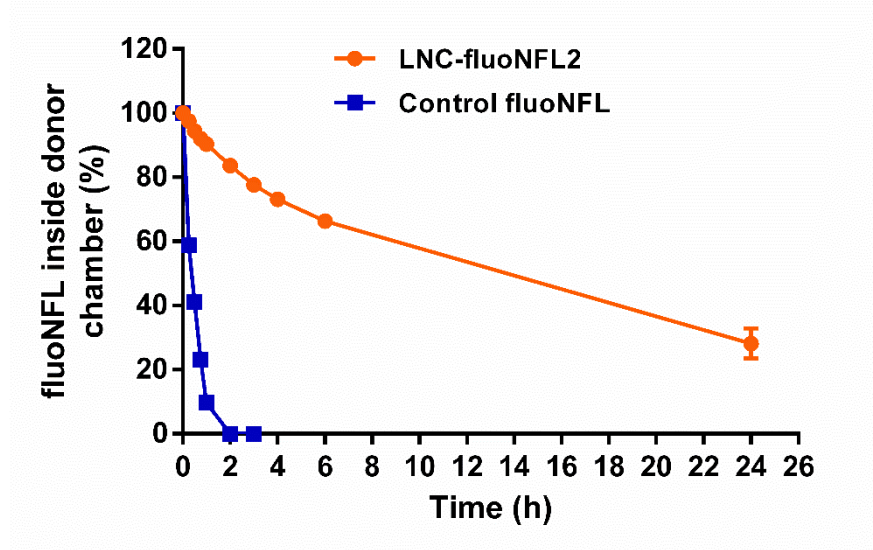
5   **Figure 3:** FluoNFL desorption kinetics from LNC-fluoNFL2 surface in 0.05 M Tris buffer pH

6   7.4 at 37 °C and 75 rpm.

7   **Complement consumption by the nanocapsules**

8   Complement consumption by the control LNCs and the fluoNFL-functionalized LNCs was

9   assessed by the CH50 assay. The particle concentration in the control LNCs was quantified by

10   NTA and was used to calculate surface area of the LNC formulations. The percentage of CH50

11   unit consumptions by the control LNCs, and the peptide functionalized LNCs were plotted

12   against surface area of the nanocapsules in 1 mL of NHS (Fig. 4). The complement consumption

13   by all three nanocapsules increased as surface area of the nanovectors increased per mL of NHS.

14   The percentage of CH50 unit consumption by control LNCs and LNC-fluoNFL1 was similar and

15   reached only 9.8 and 7.6 % respectively at around 700 cm^2/mL NHS. The complement

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

2 area.

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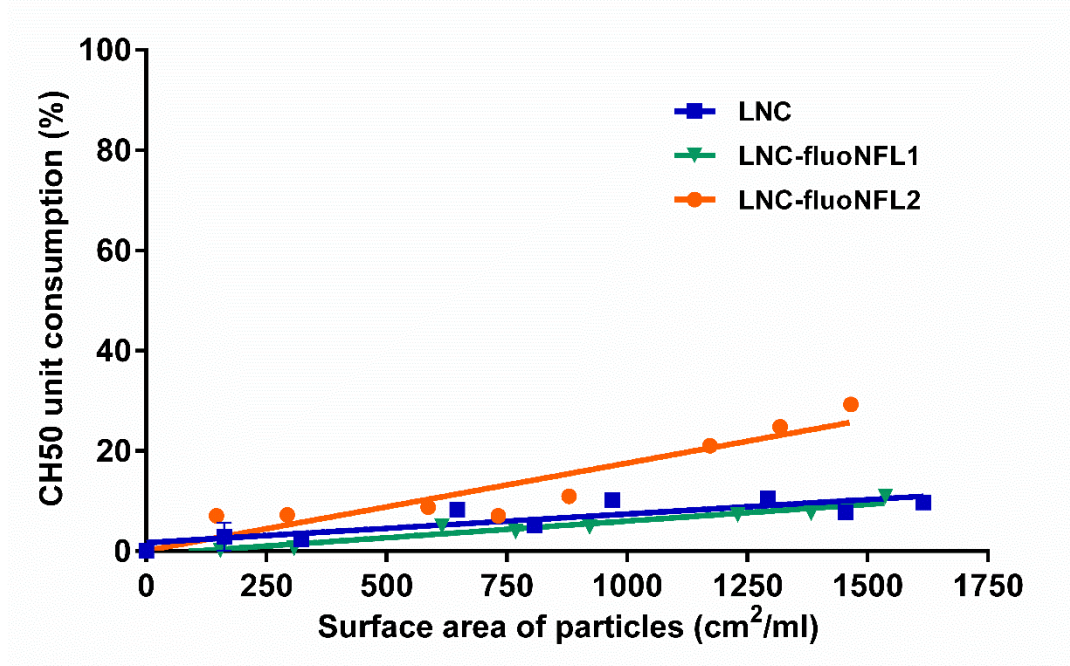
4 **Figure 4:** Complement consumption at 37 °C by control LNCs, LNC-fluoNFL1 and LNC-

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 µM 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

1 (Fig. 5). For each formulation, the cellular uptake increased as time was increased, showing the

2 time dependency of the cell internalization. The internalization of LNC-DiA was of 0.2, 0.8, 2.3

3 and 11.8 % after 30 min, 1 h, 6 h and 24 h respectively. LNC-DiA-fluoNFL1 uptake was 1.2,

4 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

5 and 86.2 % for LNC-DiA-fluoNFL2. At each time point, the cellular internalization of LNC-

6 DiA-fluoNFL2 was significantly higher compared to LNC-DiA-fluoNFL1 and LNC-DiA,

7 whereas uptake of LNC-DiA-fluoNFL1 was significantly higher compared to LNC-DiA.

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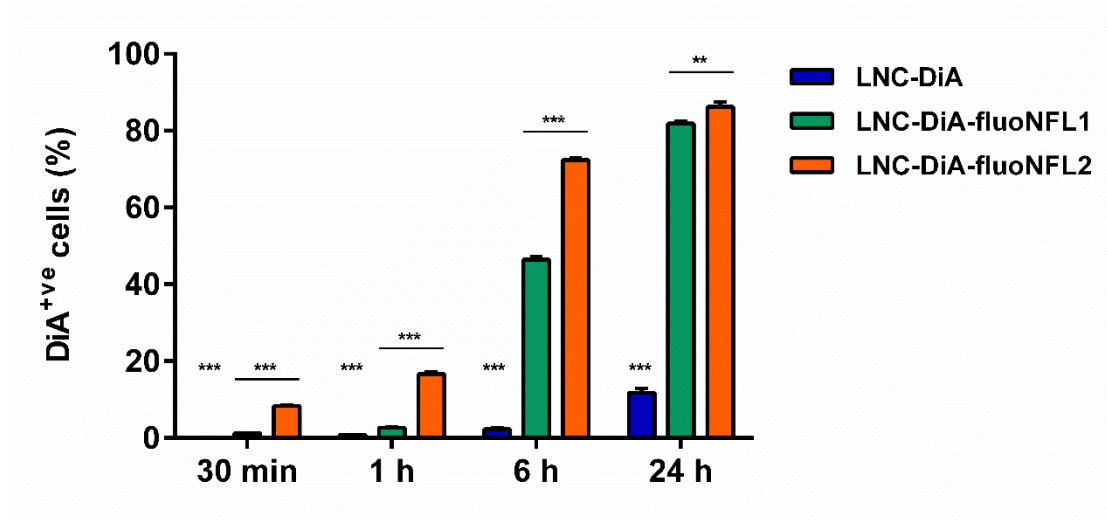
9 **Figure 5.** Enhancement of LNC internalization at 37 °C into U87MG cells with increasing

10 concentration of the fluoNFL peptide on LNC surface. The cells were incubated with 1.23

11 mg/mL of LNC-DiA, LNC-DiA-NFL1 and LNC-DiA-fluoNFL2 for 30 min, 1 h, 6 h and 24 h.

12 Twenty thousand events per sample were analyzed and percentages of DiA^{+ve} cells were

13 measured. The experiments were performed in triplicate. Statistical analysis was performed with

14 one-way ANOVA with Tukey post-hoc test ($p < 0.05$ is denoted by (*), $p < 0.01$ by (**) and p

15 < 0.001 by (***), n=3).

1   Moreover, to investigate the necessity of the peptide adsorption on LNC (during the

2   formulation of LNC-fluoNFL2) to enhance its cellular uptake, DiA-labelled LNC and fluoNFL

3   (at same peptide concentration as LNC-DiA-fluoNFL2) were mixed to prepare 'LNC-DiA &

4   fluoNFL imm. mix.' and the cells were treated immediately for 1 h at 37 °C. The uptake of the

5   immediate mixture was significantly lower (3.9-folds) compared to LNC-DiA-fluoNFL2, but

6   slightly higher compared to LNC-DiA (Fig. 6).

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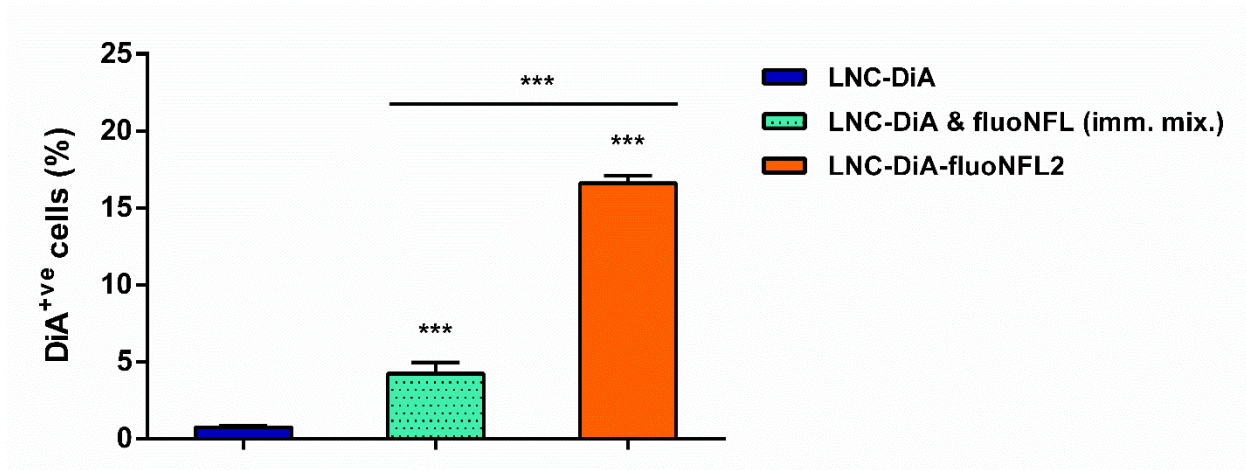
8   **Figure 6:** Internalization of LNC-DiA, immediate mixture of LNC-DiA and fluoNFL2, and

9   LNC-DiA-fluoNFL2 into U87MG cells at 37 °C after 1 h. The cells were incubated with 1.23

10 mg/mL of LNC-DiA, 'LNC-DiA & fluoNFL imm. mix.' and LNC-DiA-fluoNFL2 for 1 h.

11 Twenty thousand events per sample were analyzed and percentage of DiA^{+ve} cells was measured.

12 The experiments were performed in triplicate. Statistical analysis was performed with oneway

13 ANOVA with Tukey post-hoc test ($p < 0.05$ is denoted by (*), $p < 0.01$ by (**) and $p < 0.001$ by

14 (***), n=3).

15 Additionally, the higher cellular internalization of LNC-fluoNFL2 compared to control LNCs

16 was visualized by confocal microscopy (Fig. 7). The cells were first treated by LNC-DiA (green

17 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.
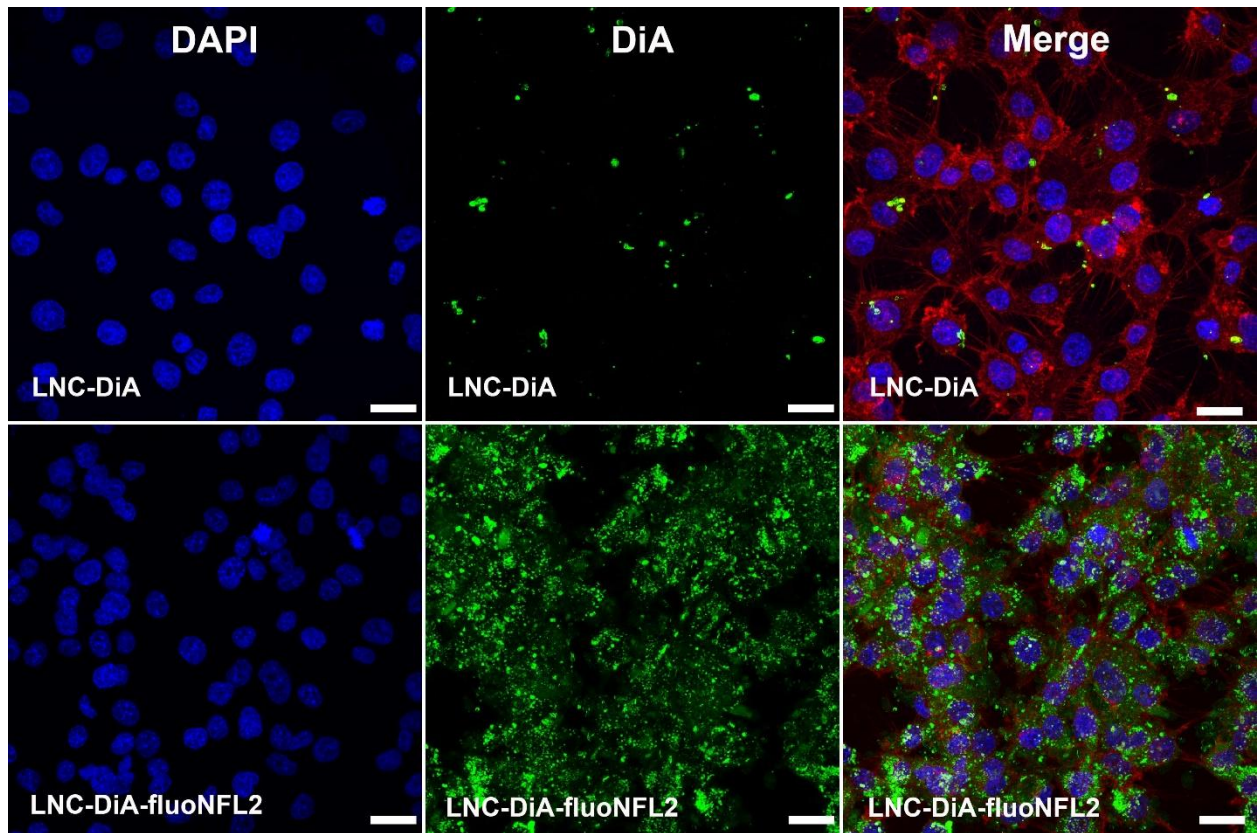
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5 **Figure 7.** Representative confocal microscopy images of enhanced LNC internalization into

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

13

1 nanocapsules. In comparison, the control LNCs were localized predominantly on the cell surface

2 rather than inside the cytoplasm.

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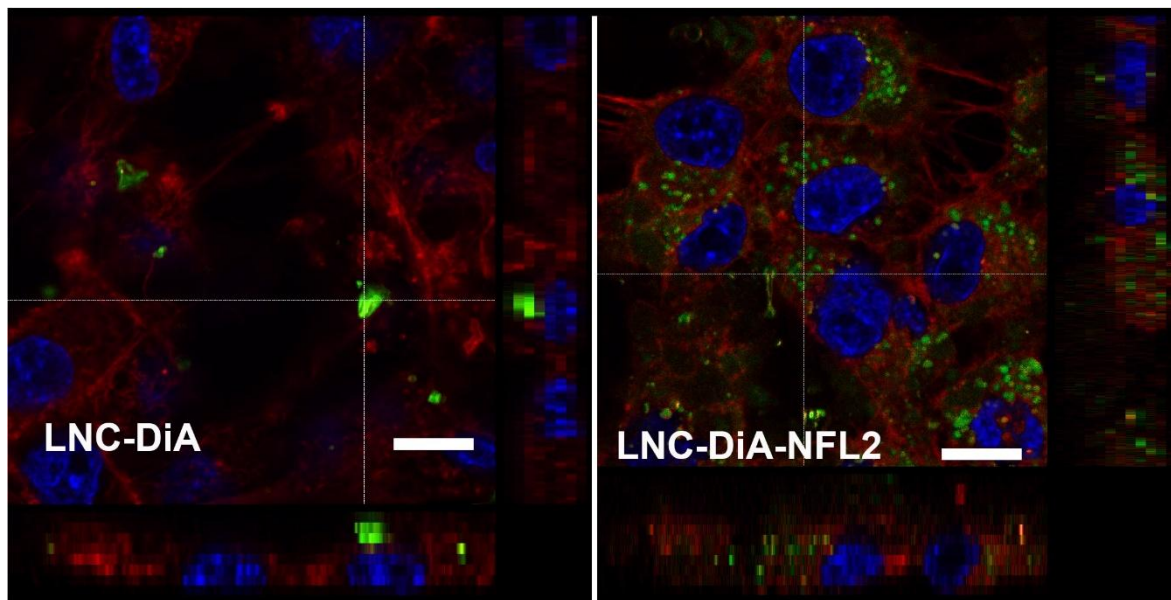
4 **Figure 8.** Orthogonal sections of stacked confocal microscopy images showing LNC

5 internalization into U87MG cells. Majority of LNC-DiA-NFL2 was localized into cell

6 cytoplasm, whereas LNC-DiA was chiefly localized on cell surface. The cells were treated at 37

7 °C for 6 h with 1.23 mg/mL of LNC-DiA and LNC-DiA-NFL2. Blue is DAPI staining (nuclei),

8 green is DiA (LNC) and red is phalloidin-TRITC staining (F-actin, cytoskeleton). White bar = 10

9 μm.

10 **Preferential accumulation of fluoNFL functionalized lipid nanocapsules in human GBM**

11 **cells compared to normal human astrocytes**

12 To inspect the targeting capability of the fluoNFL-functionalized LNC towards human GBM

13 cells, internalization of DiA loaded LNC and LNC-fluoNFL2 into NHA were measured and

14 compared with the uptake in U87MG cells. The internalization of LNCs was significantly higher

15 in Normal Human Astrocytes (NHA) compared to U87MG cells at 1 h and 6 h (Fig. 9). Surface-

16 functionalization with the fluoNFL peptide significantly enhanced the uptake of LNCs in NHA

1 by 5.2-folds and 3.5-folds at 1 h and 6 h respectively, compared to control LNCs (LNC-DiA)

2 (Fig. S4). In contrast, LNC functionalization with fluoNFL enhanced the LNC uptake into

3 U87MG cells by 21.6-folds and 31.5-folds at 1 h and 6h respectively, compared to control LNCs.

4 Although there was no significant difference of LNC-DiA-fluoNFL2 internalization in NHA and

5 U87MG cells at 1 h, the uptake was significantly higher (4.4-folds) in the GBM cells by 6 h.

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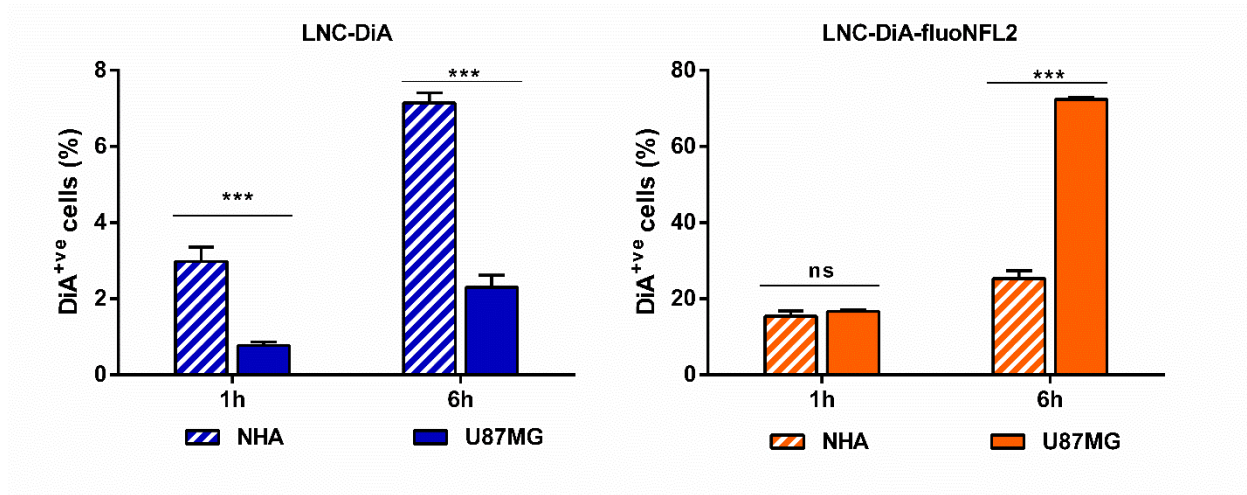
7 **Figure 9:** Higher LNC-DiA internalization into NHA compared to U87MG cells, whereas LNC-

8 DiA-fluoNFL2 was internalized preferentially into U87MG cells compared to NHA. The cells

9 were incubated with 1.23 mg/mL of LNC-DiA or LNC-DiA-fluoNFL2 for 1 h and 6 h. Twenty

10 thousand events per sample were analyzed and percentages of DiA^{+ve} cells were measured. The

11 experiments were performed in triplicate. Statistical analysis was performed with t-test (p <0.05

12 is denoted by (*), p <0.01 by (**) and p <0.001 by (***), n=3). Supporting Information: Fig. S4.

13 **Mechanisms of fluoNFL-functionalized lipid nanocapsule internalization in U87MG**

14 **human glioblastoma cell**

15 To evaluate the possible mechanism of LNC-fluoNFL2 internalization in U87MG cells, the

16 cells were treated with the DiA-labelled nanocapsules in different energetic conditions *i.e.* at 4°C

17 and ATP-depleted conditions for 1 h and 6 h (Fig. 10a). At 4°C, the internalization of LNC-DiA-

1   fluoNFL2 was almost completely stopped both at 1 h and 6 h and therefore the alteration was

2   significant compared to the normal conditions (n.c.). In ATP-depleted conditions, LNC-DiA-

3   fluoNFL2 uptake was near 0 % after 1 h, but increased to about 25 % of the n.c after 6 h. At 1 h,

4   the LNC-DiA-fluoNFL2 uptake was similar in both conditions (4 °C and ATP-depleted), but

5   significantly different after 6 h. Internalization of fluoNFL solution was significantly and

6   similarly reduced at 4°C and in ATP-depleted condition at 1 h compared to n.c. (Fig. 10b).

7   Therefore, LNC-fluoNLF2 uptake in U87MG cells was temperature and energy-dependent

8   process, similarly to fluoNFL as previously reported.[18]

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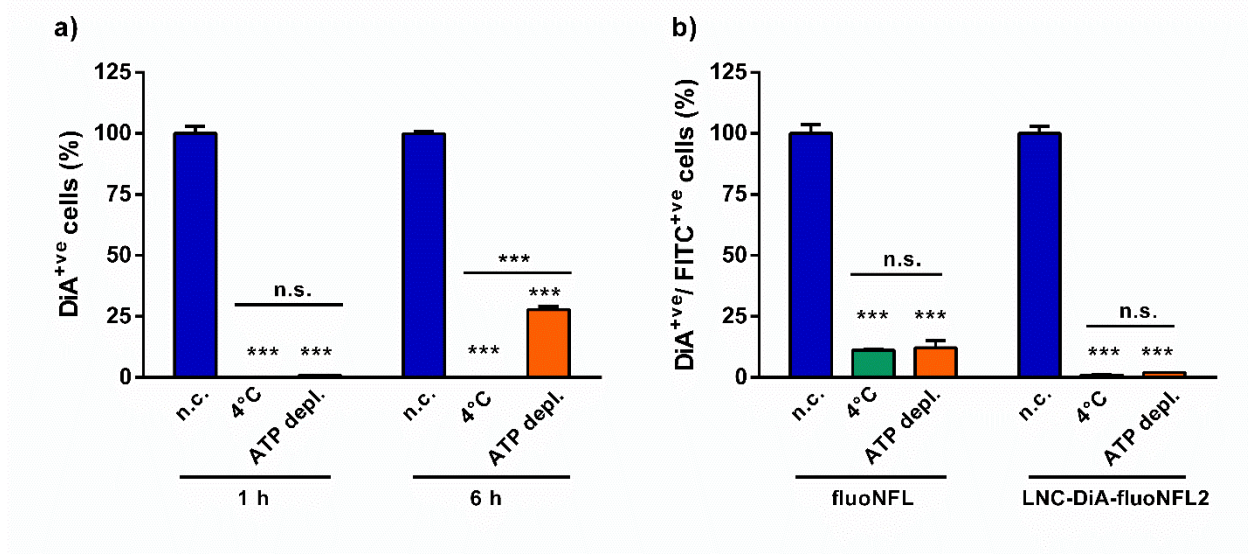
10   **Figure 10:** a) LNC-DiA-fluoNFL2 internalization into U87MG cells at different energetic

11   conditions. The cells were incubated with 1.23 mg/mL of LNC-DiA-fluoNFL2 for 1 h and 6 h at

12   37 °C (n.c.), 4°C and ATP-depleted conditions. b) Comparison of internalization of fluoNFL and

13   LNC-DiA-fluoNFL2 in U87MG cells at different energetic conditions at 1 h. Twenty thousand

14   events per sample were analyzed and percentages of DiA^{+ve} cells (for LNC-DiA-fluoNFL2) or

15   FAM^{+ve} cells (for fluoNFL) were measured. The experiments were performed in triplicate.

1 Statistical analysis was performed with oneway ANOVA with Tukey post-hoc test (p <0.05 is

2 denoted by (*), p <0.01 by (**) and p <0.001 by (***), n=3).

3   To further evaluate the possible uptake pathway(s) of the peptide-functionalized LNCs,

4 exclusion of particular endocytosis mechanisms was achieved by using inhibitors of the foremost

5 endocytosis pathways. The cells were pretreated for 30 minutes with different inhibitors

6 followed by 1 h treatment with the LNC-DiA-NFL2. LNC uptake was significantly inhibited in

7 presence of each of these inhibitors (Fig. 11a). LNC-DiA-NFL2 internalization was the lowest in

8 presence of DAM, followed by CP, MβCD and PMA. A strong correlation between fluoNFL

9 internalization and LNC-DiA-fluoNLF2 uptake was observed (Fig. 11b). Like the

10 functionalized-LNC, fluoNFL uptake was most strongly inhibited by DAM, followed by similar

11 inhibition in presence of CP and MβCD, and lastly PMA.

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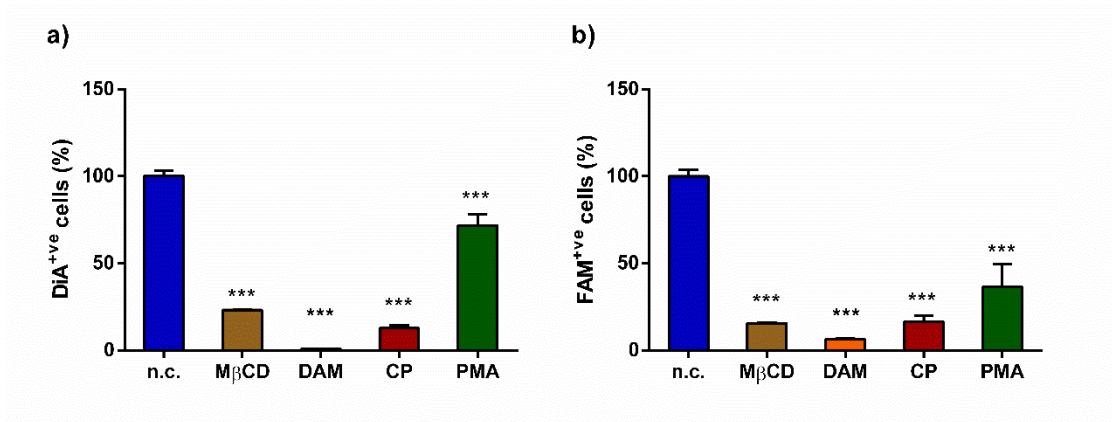
13 **Figure 11:** Internalization of LNC-DiA-fluoNFL2 (a) and fluoNFL (b) into U87MG cells at 37

14 °C (n.c.) after 30 min pretreatment with various inhibitors (MβCD, DAM, CP and PMA)

15 followed by 1 h incubation with the nanocapsule (1.23 mg/mL) or the peptide solution

16 (equivalent fluoNFL concentration of LNC-DiA-fluoNFL2). Twenty thousand events per sample

17 were analyzed and percentages of DiA^{+ve} cells (for LNC-DiA-fluoNFL2) or FAM^{+ve} cells (for

18 fluoNFL) were measured. The experiments were performed in triplicate. Statistical analysis was

1   performed with oneway ANOVA with Dunnett's post-hoc test (p <0.05 is denoted by (*), p

2   <0.01 by (**) and p <0.001 by (***), n=3).

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

4   To evaluate the *in vitro* efficacy of FcTriOH loaded LNC formulations, cell viability was

5   evaluated by MTS assay after 72 h of treatment with the formulations (Fig. 12). The IC_{50} of

6   FcTriOH solution was of 1.31 µM which was slightly reduced to 1.05 µM when the drug was

7   loaded in LNC. However, the peptide-functionalized LNC-FcTriOH-NFL2 had the lowest IC_{50}

8   of 0.46 µM, which was 2.8-folds and 2.3-folds lower compared to the drug solution and the drug-

9   loaded non-functionalized LNCs (LNC-FcTriOH). The blank LNCs showed toxicity at much

10   higher concentrations (IC_{50} 1.42 mg/mL) compared to the drug-loaded LNCs (Supporting

11   information: Fig. S1). The fluoNFL solution did not show any toxicity in the tested

12   concentrations (Supporting information: Fig. S2).

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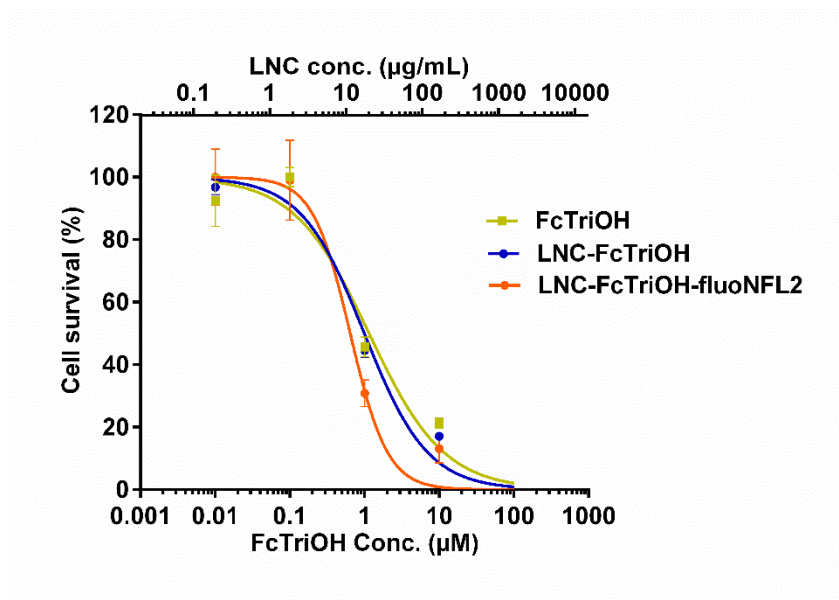
14   **Figure 12.** Survival percentages of U87MG cells after 72 h treatment with various

15   concentrations of LNCs (LNC, LNC-FcTriOH and LNC-FcTriOH-NFL2) and FcTriOH solution.

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4   **Preliminary *in vivo* study in ectopic tumor xenograft model**

5   Nude NMRI mice were subcutaneously inoculated with human U87MG cells to acquire

6   preliminary knowledge of possible tumor reduction efficacy and toxicity of the developed

7   formulations after i.v. administration. After 7 days of cell implantation, the average tumor

8   volume was around 70 mm^3 and the animals were divided into five groups (saline, LNC-blank,

9   LNC-blank-fluoNFL2, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2) and injected intravenously

10   with 70 µL of treatments (20 mg FcTriOH per kg body weight, or 822.4 mg LNCs with/without

11   21.5 mg peptide per kg of body weight) on day 7 and day 10 (Fig. 13). The relative tumor

12   volume of the saline and the LNC-blank treated mice gradually increased from day 7 until the

13   end of the study, whereas it was stable until day 17 and then increased for LNC-blank-fluoNFL2

14   treated group. For the FcTriOH treated groups (LNC-FcTriOH and LNC-FcTriOH-fluoNFL2),

15   relative tumor volume gradually decreased up to day 17, remained smaller than their initial

16   volume (at the first treatment injection day) up to day 22, and then increased gradually.

17   Compared to saline treated group, the relative tumor growth for LNC-FcTriOH and LNC-

18   FcTriOH-fluoNFL2 treated groups were significantly lower (40.1 and 44.2 % lower respectively)

19   at day 17 of the study (Fig. 13). This significant difference was maintained up to day 22 of the

20   study, and was absent afterwards.

21   None of the mice showed any immediate or delayed behavioral signs of pain or toxicity after

22   the treatments were administered. Moreover, they were growing gradually as evident from their

23   relative weight which increased about 20 % at the end of the study period (day=50): Fig. 13.
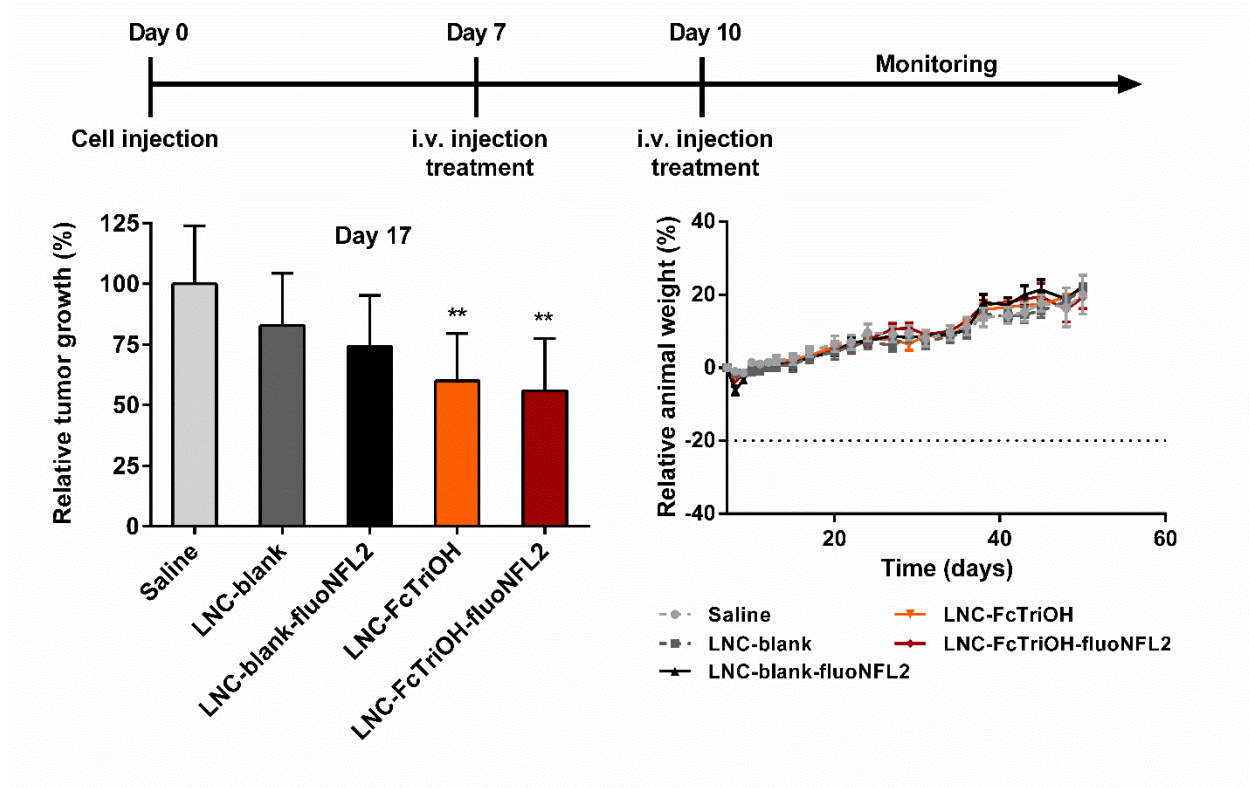
Figure 13: Relative tumor growth (on day 17) and relative animal weight of subcutaneous U87MG human glioblastoma tumor bearing mice. Each mouse was injected with 2×10^6 cells (in 50 μL PBS) in the right flank on day 0 of the study. As the average tumor volume reached about 70 mm^3 after one week, the 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 (**) and $p < 0.001$ 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

20

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 μM.[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 nm, whereas diameter of the LNC-fluoNFL1 (NML

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 nm^2 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 nm^2 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 Kolliphor [19]

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

1   Waal's forces) and therefore resisted the impact of high NaCl concentrations, but loses its

2   significant size difference with control LNCs in higher Tris concentrations. To evaluate if the

3   fluoNFL will be rapidly removed from the LNC surface after dilution, LNC-fluoNFL2 was

4   dialyzed (MWCO 100 kD dialysis bag) against 1x Tris buffer solution at 37 °C and 75 rpm. Free

5   peptide concentration was quantified from the receiver compartment by HPLC. Desorption of the

6   fluoNFL from LNC surface was slow and gradual and only 33.6 % peptide was desorbed after 6

7   h (Fig. 3). Moreover, NFL-functionalized LNCs was reported to maintain their characteristics in

8   cell culture medium.[22] Therefore, the LNC-fluoNFL2 formulation can be promising for

9   administration by i.v. injection. Additionally, this dialysis experimentation also indicated a high

10 percentage of fluoNFL adsorption in LNC-fluoNFL2. About 94.4 % and 90.4 % of the added

11 peptide were remaining in the dialysis chamber after 30 min and 1 h dialysis respectively for

12 LNC-fluoNFL2, whereas it was only 41.1 % and 9.6 % for the control fluoNFL solution (same

13 initial concentration as LNC-fluoNFL2). Theoretically, up to 58.9 % of the added 3 mM peptide

14 in LNC-fluoNFL2 should be able to cross the dialysis membrane to reach the receiver chamber

15 after 30 min dialysis, if they were free. Therefore, this result also showed that the peptide

16 adsorption percentage in LNC-fluoNFL2 was possibly very high and strongly attached to the

17 LNC surface and further study is necessary to understand the real mechanism.

18   As the size and zeta potential of the LNCs was altered after fluoNFL adsorption, it could

19 impact the *in vivo* fate of the nanocarrier. Enhanced particle size and positive zeta potential may

20 significantly increase complement protein consumption by nanoparticles, leading to rapid

21 removal from systemic circulation by the mononuclear phagocytic system (MPS).[34-35] The CH50

22 unit consumption by the LNC-fluoNFL1 was similar to what shown by the control LNCs (Fig.

23 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 μM 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

1 fluorescence around 660 nm that can be detected in FACS.[39-40] However, maximum 0.1 % dead

2 cells were detected in the FACS samples which confirms that the LNC concentration used for

3 treatment of cells was non-toxic. The internalization of all three formulations increased with time

4 (Fig. 5). At each time point, the uptake of LNC-DiA-fluoNFL2 was significantly higher

5 compared to LNC-DiA-fluoNFL1 and LNC-DiA, whereas the internalization of LNC-DiA-

6 fluoNFL1 was significantly higher compared to LNC-DiA. It was also observed that the peptide

7 needs to be absorbed onto the LNC surface (by 24 h stirring) for maximizing LNC

8 internalization as the uptake of 'LNC-DiA and fluoNFL immediate mixture' was significantly

9 lower compared to LNC-DiA-fluoNFL2 (Fig. 6). Therefore, the internalization of nanocapsules

10 into U87MG cells is dependent on the concentration of NFL on LNC surface. Confocal

11 microscopy images visually confirmed the much higher cellular uptake of LNC-DiA-fluoNFL2

12 compared to LNC-DiA (Fig. 7), and showed that majority of the NFL-functionalized LNCs was

13 into the cytoplasm whereas the LNC-DiA was mostly attached to the cell membrane (Fig. 8). It

14 has been shown for the first time that the NFL peptide concentration (as a targeting-ligand) onto

15 nanocarrier surface can have significant impact on the rate and the extent of the nanovector

16 cellular internalization, on human GBM cells. Therefore, this strategy could be used to improve

17 nanocarrier targeting efficiency to other type of cells in which the peptide can efficiently enter

18 *i.e.* brain neural stem cells.[18, 22, 41]

19 Previously, Paillard *et al.* reported that the internalization of untargeted LNCs was not

20 preferentially targeted into GBM cells and entered also healthy astrocytes.[38] Additionally,

21 surface-functionalization with the fluoNFL peptide significantly enhanced the uptake of LNCs in

22 NHA, compared to control LNCs (LNC-DiA), NFL being a cell penetrating peptide. Therefore,

23 to investigate the targeting capacity of the LNC-fluoNFL2 towards U87MG cells, LNC-DiA and

1   LNC-DiA-fluoNFL2 were incubated with NHA and their cellular uptake after 1 h and 6 h was

2   measured and compared with their uptake in U87MG cells (Fig. 9). At 1 h, no significant

3   difference was observed between LNC-fluoNFL2 internalization in NHA and U87MG cells.

4   However, the rate of LNC-fluoNFL2 internalization was much faster in the GBM cells and the

5   nanovector entered significantly more in the cancer cells compared to NHA. Therefore, the

6   cellular internalization of LNC-fluoNFL2 was preferentially targeted towards the human GBM

7   cells compared to healthy cells.

8   To investigate the possible pathway(s) of LNC-fluoNFL2 internalization in U87MG cells, its

9   uptake was followed in different energetic conditions and in presence of various endocytosis

10   pathway inhibitors. The internalization of the nanocarrier was significantly reduced (compared to

11   37 °C) when incubated at different energetic conditions (4 °C and ATP-depleted condition) (Fig.

12   10a). In ATP-depleted conditions, no LNC-DiA-fluoNFL2 uptake was showed after 1 h, but an

13   increase to about 25 % of the n.c after 6 h was observed. On the contrary, at 4°C, no LNC-DIA-

14   fluoNFL2 was internalized after 1 h and 6 h of incubation. This result could suggest that in ATP-

15   depleted conditions, after 6h, these nano-objects were able to be internalized via an independent-

16   energy pathway. But the same result was surprisingly not observed at 4°C. Indeed, a higher

17   rigidity and a lower permeability of lipid bilayer have been demonstrated at low temperature and

18   could explain this difference [42].

19   Thus, the LNC-fluoNFL2 uptake in U87MG cell was an energy-dependent active process.

20   Comparable trend was observed in cellular uptake of the fluoNFL alone (Fig. 10b) which was

21   also mentioned in previously reports.[18]

22   The dependency of cellular uptake on energy indicates that the internalization possibly occurs

23   by endocytosis. To further illustrate about the particular internalization pathway(s) involved, the

1   cells were pretreated with various inhibitors of the chief endocytosis pathways. Treatment with

2   MβCD depletes cholesterol and inhibits both clathrin- and caveolin-mediated endocytosis, DAM

3   prevents macropinocytosis, chlorpromazine blocks clathrin-dependent endocytosis and PMA

4   impedes caveolin-dependent endocytosis.[38, 43] As previously reported for the NFL peptide [23]

5   (also observed in our experiments, Fig. 11b), the internalization of the LNC-fluoNFL2 was not

6   dependent on one particular endocytosis pathway, rather on several and its uptake was

7   significantly reduced when cells were pretreated with these inhibitors (Fig. 11a). Taken together,

8   the predominant pathways involved in NFL-functionalized LNC internalization were

9   macropinocytosis, clathrin-dependent and caveolin-dependent endocytosis; similar to the free

10   peptide solution. However, the very low uptake of the non-functionalized LNC into U87MG

11   cells up to 6 h was not suitable to be used as control for evaluating its cellular uptake

12   mechanisms. Moreover, the 24 h time point has been tried for determining the possible LNC

13   internalization pathways. But the cells did not survive up to 24 h in presence of the different

14   endocytosis inhibitors and the mechanism of LNC uptake in U87MG cells could not be

15   determined by this method.

16   A promising ferrocifen-type anticancer drug FcTriOH was encapsulated in the LNCs. FcTriOH

17   belongs to the hydroxypropyl series of the acyclic ferrocifen family, possibly forms an

18   intrinsically electrophilic and cytotoxic metabolite i.e. tetrahydrofuran substituted quinone

19   methides in living cancer cells, and have shown significantly enhanced activity on hormone-

20   independent breast cancer cells compared to the acyclic ferrociphenol.[12] This was the first time

21   this compound was studied *in vivo* on a human GBM tumor model in mice. The *in vitro*

22   antiproliferative activity of FcTriOH was assessed by MTS assay. The cells were treated with

23   0.1-100 μM of FcTriOH and its formulations for 72 h. Up to 0.1 μM, cell survival was above 80

1   % for all treatment groups (Fig. 12). Between 0.1-10 μM, the cell survival percentage drastically

2   reduced for cells treated with FcTriOH, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2 resulting

3   in IC_{50} values of 1.31 μM, 1.05 μM and 0.46 μM respectively. The survival of the cells treated

4   with control LNC reduced significantly between 10 and 100 μM with an IC_{50} of 22.5 μM.

5   Corresponding concentrations of fluoNFL solution did not alter cell viability which was also

6   reported previously.[18]

7   In the preliminary *in vivo* study, an U87MG subcutaneous GBM tumor model was used to

8   evaluate potential tumor reduction efficacy or possible toxicity after two tail vein injections

9   equivalent to 20 mg/kg FcTriOH. As no previous reports about this FcTriOH administration in

10   animals were available, the dose was chosen based on previous *in vivo* studies involving other

11   ferrocifen molecules.[44] The two i.v. injections were given on day 7 and day 10. A tendency of

12   relative tumor volume gradual reduction was observed since the beginning of the treatment with

13   FcTriOH-loaded LNCs. A significant difference *i.e.* 40.1 % and 44.2 % lower relative tumor

14   volume for LNC-FcTriOH and FcTriOH-fluoNFL2 respectively, compared to the saline treated

15   group, was observed on by day 17 (Fig. 13) which was maintained up to day 22. The mouse

16   showed no behavioral signs of pain or irritation immediately after the injection. Additionally, the

17   weight of the mice never reduced and signs of pain and distress were not observed throughout

18   the study. However, the tumor reduction effect of the FcTriOH treatments was not observed from

19   day 24 (two weeks after the last injection). The tumor rapidly grew back and no significant

20   difference in relative tumor volume was observed. This possibly occurs as the drug is eliminated

21   resulting its antiproliferative effect to fade and the tumor to grow back. In fact, several

22   preclinical studies have used much higher number (6 to 20) of i.v. injections [44-45] and observed a

23   significant difference on tumor growth. In clinical practice, chemotherapy is generally

1   administered in several cycles; a treatment period followed by a waiting period for the patient to

2   wash-out and recover from the side effect of the drug. The cycle frequencies are optimized

3   depending on the treatment used. In future studies, the number of injections and/or dose should

4   be increased to possibly achieve tumor regression after FcTriOH-loaded LNC treatment.

5   Although the relative tumor volume of LNC-FcTriOH treated and LNC-FcTriOH-fluoNFL2

6   treated groups on day 17 was significantly lower from saline treated groups, the difference

7   among themselves were not significant. However, the average value of relative tumor growth

8   was slightly lower for NFL-functionalized LNC treated groups. The tendency may be more

9   clearly observed if more injections are given in the future studies.

10   The NFL-peptide concentration of LNC surface can be further increased for additional

11   enhancement of its internalization in human GBM cells. However, the currently used preparation

12   technique is not suitable for this purpose as precipitates were observed in fluoNFL peptide

13   solutions above 3 mM, probably due to its aqueous solubility limit. Additionally, covalent

14   coupling of NFL at the distal end of long-chain PEG has been described to hamper its GBM-

15   targeting properties.[19] However, addition of NFL solution/suspension above 3 mM in LNC

16   dispersions resulted a viscous and hydrogel like formulation (data not shown), which could be

17   interesting for local application in the cavity after surgical removal of the tumor.[46]

18

19   **EXPERIMENTAL**

20   **Materials**

21   Macrogol 15 hydroxystearate (Kolliphor® HS15) was purchased from BASF (Germany).

22   Hydrogenated phosphatidylcholine from soybean (Lipoid S PC-3) was provided from Lipoid

23   GmbH (Germany), caprylic/capric triglycerides (Labrafac Lipophile WL1349) was supplied by

1   Gattefosse (France). FcTriOH was provided by PSL Chimie ParisTech (France), Feroscan SME.

2   5,6-Carboxyfluorescein labelled NFL.TBS-40.63 peptide (fluoNFL) was purchased from

3   Polypeptide Laboratories (France).

4   The human glioblastoma cell line U87MG was collected from ATCC (USA). Normal human

5   astrocytes (NHA), astrocyte basal medium (ABM), SingleQuots^{TM} kit supplements & growth

6   factors, L-glutamine, penicillin-streptomycin and Dulbecco's modified Eagle's medium with 1

7   g/L L-glucose (DMEM) were provided by Lonza (France). Methyl-β-cyclodextrin (MβCD), 5-

8   (N,N-dimethyl) amiloride hydrochloride (DAM), chlorpromazine (CP), phalloidin–

9   tetramethylrhodamine-B-isothiocyanate (phalloidin-TRITC), sodium azide and 2-deoxy-D-

10   glucose were purchased from Sigma (Germany). Phorbol-12-myristate-13-acetate (PMA) was

11   collected from Abcam (France). 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide)

12   (DiA), 4',6-diamidino-2-phenylindole (DAPI), Trypsin-EDTA 1x, non-essential amino acids

13   solution 100x (NEAA), fetal bovine serum (FBS) and ProLong Gold antifade were collected

14   from Thermo Fisher Scientific (USA). 3-carboxymethoxyphenyl-2-(4-sulfophrenyl)-2H-

15   tetrazolium (MTS) and phenazine methosulfate (PMS) was purchased from Promega (USA).

16   Normal human serum (NHS) was provided by the "Etablissement Français du Sang" (Angers,

17   France). Sheep erythrocytes and hemolysin were purchased from Eurobio (France). Sodium

18   chloride (NaCl) was purchased from Prolabo (Fontenay-sous-bois, France). Ultra-pure water

19   (UPW) was obtained from a Millipore filtration system. All the other reagents and chemicals

20   were of analytical grade.

21

22   **Preparation of lipid nanocapsules**

23   ***Preparation of stock lipid nanocapsules***

1   Stock LNC (LNC-stock) was prepared using phase inversion temperature technique.[21] In brief,

2   Kolliphor® HS15 (16.9 % w/w), Lipoid® S PC-3 (1.5 % w/w), Labrafac Lipophile WL1349

3   (20.6 % w/w), NaCl (1.8 % w/w) and UPW (59.2 % w/w) were mixed under magnetic stirring at

4   60 °C for 15 min. Three heating-cooling cycles were performed between 90 °C and 60 °C.

5   During the last cooling step, when the temperature was in the phase inversion zone (78-83 °C),

6   ice-cold UPW was added (final concentration 88.4 % w/w) to induce irreversible shock and form

7   the LNC-stock. The nanocapsules were then passed through 0.2 µm cellulose acetate filter to

8   remove any aggregates and stored at 4 °C.

9   DiA-labelled stock LNC was prepared by incorporating 0.1% (w/w) DiA in the formulation at

10   the first step with other excipients.

11

12   ***Preparation of fluoNFL functionalized lipid nanocapsules and lipid nanocapsules***

13   1 mL of stock LNC (LNC-stock) was stirred at room temperature for 24 h with 0.369 mL of 1

14   mM (0.86 % w/w) (similar to [19, 22]) or 3 mM (2.57 % w/w) fluoNFL solution (in water) to

15   prepare the fluoNFL functionalized LNCs (LNC-fluoNFL1 and LNC-fluoNFL2 respectively).

16   Similarly, 1 mL of the LNC stock was stirred at room temperature for 24 h with 0.369 mL of

17   UPW to produce final control LNCs.

18   The DiA (0.1 % w/w) labelled LNCs was also functionalized with the fluoNFL by same

19   method as LNC-fluoNFL1 and LNC-fluoNFL2.

20

21   ***Preparation of FcTriOH-loaded lipid nanocapsules***

22   The FcTriOH-loaded LNC (LNC-FcTriOH) was prepared similarly as control LNCs, except

23   FcTriOH (0.9 % w/w) was added at the first step of the formulation with the other excipients.

1 Subsequently, fluoNFL was adsorbed at their surface using identical method as LNC-fluoNFL2

2 to produce drug loaded NFL-functionalized LNCs.

3

4 ***Optimization of lipid nanocapsules for in vivo studies***

5 For *in vivo* studies, the amount of ice-cold UPW used to induce shock to produce the LNCs

6 was adjusted (final concentration 70.9% w/w) to produce concentrated LNCs according to

7 previously published article.[24] NaCl concentration was also adjusted to keep the final

8 formulations isotonic with blood.

9

10 **Characterization of the lipid nanocapsules**

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

12 The mean diameter and polydispersity index (PDI) of the LNCs were determined by dynamic

13 light scattering (DLS) technique using Zetasizer Nano ZS (Malvern Instruments Ltd, UK). The

14 LNCs were diluted 100-folds in UPW before the analysis. The measurements were performed at

15 backscatter angle of 173°. The measured average values were calculated from 3 runs, with 10

16 measurements within each run.

17 Zeta potential of the nanocarriers was measured using laser Doppler micro-electrophoresis

18 using Zetasizer Nano ZS (Malvern Instruments Ltd, UK).

19 Additionally, the particle concentration in the control LNC dispersion was determined using

20 nanoparticle tracking analysis (NTA) as described previously.[37] The NTA was carried out using

21 the NanoSight NS300 (Malvern Instruments Ltd, UK). Briefly, the NDDS samples were diluted

22 to optimum concentrations with UPW and were infused in the sample chamber using a syringe

23 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.

5

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 μm, 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 → 55:25, 25 min). Flow rate was 1 mL/min, injection volume

15 was 10 μL 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 μg/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 μg/mL. To quantify unencapsulated drug concentration,

1. formulations were placed on centrifugal concentrator devices with polyethersulfone membrane

2. (MWCO 30 kD, Amicon Ultra-500, Millipore) and centrifuged at 4000 g for 30 minutes to

3. separate the free drug from the rest of the formulation. The filtrates containing unencapsulated

4. drug were collected and ethanol (2-folds) was added to solubilize any undissolved drug. Drug

5. dosage in the above-mentioned samples was performed in a HPLC system (Waters, France). A

6. C18 analytical column (250 x 4.6 mm, 5 μm, Waters, France) was used at room temperature.

7. UPW and acetonitrile (45:55, v/v) were used as mobile phases. Flow rate was 1 mL/min,

8. injection volume was 10 µL and FcTriOH was detected at 304 nm. Analysis of the data was

9. performed by Empower 3 software (Waters). Retention time of FcTriOH was 8.1 min.

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

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

11. Drug loading was calculated using the following equation:

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

12.

13. ***Interaction between LNCs and fluoNFL***

14. The LNC-fluoNFL2 and control LNCs were diluted in UPW or in various concentrations

15. (0.005, 0.05, 0.15, 0.25, 0.5 and 1 M) of NaCl or Tris buffer, incubated for 30 minutes before

16. measuring their size by DLS.[22]

17. Additionally, LNC-fluoNFL2 and control fluoNFL solutions were taken in dialysis bags

18. (MWCO 100 kD, Spectra/Por® biotech grade cellulose ester membrane, SpectrumLabs,

19. Netherlands) and dialyzed against Tris buffer (0.05 M, pH 7.4) at 37°C, stirred at 75 rpm. At

20. various time points (0.25, 0.5, 0.75, 1, 2, 3, 4, 6 and 24 h) samples were collected from the

1   receiver chamber and amount of the free peptide was quantified using the HPLC method

2   mentioned above.

3

4   **Complement consumption assay (CH50 assay)**

5   The residual hemolytic capacity of NHS towards antibody-sensitized sheep erythrocytes after

6   incubation with different LNC formulations was measured to evaluate the complement activation

7   by the formulations.[47] In brief,[37] aliquots of NHS were incubated with increasing concentrations

8   of the LNCs at 37 °C for 1 h. Subsequently, the different volumes of the NHS were incubated

9   with a fixed volume of hemolysin-sensitized sheep erythrocytes at 37 °C for 45 min. The volume

10 of serum that can lyse 50 % of the erythrocytes was calculated ("CH50 units") for each sample

11 and percentage of CH50 unit consumption relative to negative control was determined as

12 described previously.[34] Particle number in the LNC dispersion was determined by NTA and

13 nanocarrier concentration per mL of NHS was calculated according to following equation:

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

14 Subsequently, surface area of the NDDSs per mL of NHS was calculated according to the

15 following equation:

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

16 The CH50 unit consumption by the different LNCs was compared by plotting the percentage

17 of CH50 unit consumption as a function of their surface area.

18

19 **Cell culture**

20 The human glioblastoma cell line U87MG was cultured at 37 °C under 5 % CO_2 in DMEM

21 supplemented with 10 % FBS, 5% L-glutamine, 5 % NEAA and 5 % penicillin-streptomycin.

1   NHA was cultured at 37 °C under 5 % CO_2 in ABM supplemented by the 'AGM SingleQuot^{TM}

2   Kit'. The cells were passaged once they were about 70 % confluence.

3

4   **Flow cytometry**

5   ***Kinetics of LNC internalization in U87MG cells***

6   The kinetics of internalization of the DiA-labelled LNCs (LNC-DiA, LNC-DiA-fluoNFL1 and

7   LNC-DiA-fluoNFL2) in U87MG cells was assessed using the BD FACSCanto™ II flow

8   cytometer (BD Biosciences). In brief, cells were seeded in 6-well plates at 5×10^5 cells/well

9   concentration for 24 hours. Subsequently, they were treated with the different DiA-labelled

10   LNCs (1.23 mg/mL) for 0.5, 1, 6 and 24 h. Afterwards, the cells were washed three times with

11   ice-cold phosphate buffer saline 1x (PBS), detached by incubating 5-10 minutes with Trypsin-

12   EDTA 1x. The cells were then centrifuged at 2000 rpm for 5 minutes, the supernatant was

13   aspirated and the cell pellet was re-dispersed in PBS. The centrifugation and re-dispersion cycle

14   was repeated twice more. Finally, the cells were suspended in trypan blue (final trypan blue

15   concentration 0.12% w/v) and the percentage of DiA positive (DiA^{+ve}) or FAM positive

16   (FAM^{+ve}) cells were analyzed by the flow cytometer. Each experiment was performed in

17   triplicate and 20,000 events per sample were analyzed in each experiment.

18

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

20   ***cells***

21   To assess the targeting-capability of the fluoNFL-functionalized LNC towards GBM cells

22   compared to healthy cells, NHA was treated for 1 h and 6 h with LNC-DiA-fluoNFL2 (method

1   mentioned above) at 37 °C and percentage of DiA^{+ve} cells was measured using the above-

2   mentioned method, and compared with the results of U87MG cells.

3

4   ***Mechanism of fluoNFL-functionalized LNCs internalization in U87MG cells***

5   To evaluate the dependency of NFL-functionalized LNC cellular internalization on energy,

6   U87MG cells were pre-incubated for 30 min at 4 °C or pretreated for 30 minutes at 37 °C (NaN$_3$

7   10 mM and 2-deoxy-D-glucose 6 mM) to deplete cellular ATP [23]. Subsequently, the cells were

8   treated for 1 and 6 h with the LNC-DiA-fluoNFL2 and percentage of DiA^{+ve} cells were measured

9   by the above-mentioned method.

10   To investigate the possible pathways of LNC-DiA-fluoNFL2 internalization in U87MG, cells

11   were pretreated with different inhibitors (MβCD 10 mg/mL, DAM 1 mM, CP 50 μM and PMA

12   10 μg/mL) for 30 min at 37 °C [23] followed by 1 h treatment with the nanocarrier and percentage

13   of DiA^{+ve} was quantified.

14   In all the above-mentioned conditions (37 °C, pre-incubation at 4 °C, pre-treatment for ATP

15   depletion, and pre-treatment with various inhibitors), internalization of fluoNFL (at equivalent

16   concentration of LNC-DiA-fluoNFL2) in U87MG cells was assessed by measuring FAM^{+ve} cells

17   to assess if the fluoNFL by itself regulates the internalization of NFL-functionalized LNC.

18

19   ***Confocal microscopy***

20   To visualize the potential effects of the fluoNFL peptide on LNC internalization, U87MG cells

21   were seeded (3×10^4 cells/well) in 24 well plates containing coverslips and incubated at 37 °C

22   for 72 h (medium was carefully replaced every 24 h) to allow the cells to grow on the coverslips.

23   Subsequently, the cells were treated with 1.23 mg/mL of LNC-DiA or LNC-DiA-fluoNFL2 for 1

1   h and 6 h at 37 °C. Afterwards, the cells were washed three times with PBS and fixed with 4 %

2   paraformaldehyde for 20 min at room temperature. Then, the cells were washed twice with PBS

3   and permeabilized by incubation with 0.1 % Triton X-100 for 10 min. The cells were washed

4   twice with PBS and incubated with 0.7 μM of phalloidin-TRITC for 1 h at room temperature.

5   Subsequently, the cells were washed twice with PBS and incubated with 3 μM DAPI for 10

6   minutes. Finally, the cells were washed 3-times with PBS and the coverslips were mounted using

7   ProLong Gold antifade mounting medium. The cells were then visualized and images were

8   captured by a confocal microscope (LSM 700 Zeiss). DAPI was excited with a 405 nm

9   wavelength and recorded at 409-453 nm (blue channel), DiA and fluoNFL were excited with a

10   458 nm wavelength and recorded at 558-666 nm (green channel) whereas TRITC was excited

11   with a 561 nm wavelength and recorded at 564-632 nm (red channel).

12

13   ***In vitro efficacy***

14   Viability of the U87MG cells to various LNC treatments was assessed by MTS assay.[19] In

15   brief, the U87MG cells were seeded in 96 well plates (5000 cells/well) and incubated for 24 h.

16   Then the medium was replaced with various concentrations of LNCs (60-600,000 folds diluted in

17   DMEM) (LNC-blank, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2), FcTriOH (22-220,000

18   folds dilution in DMEM) and fluoNFL (125-4,983,383 folds dilution in DMEM) and treated for

19   72 hours at 37 °C. After that, each well content was replaced with 100 μL of fresh DMEM.

20   Additionally, 20 μL of MTS-PMS (20:1) mixture was added in each well and incubated at 37 °C

21   for 2 h. Absorbance of the samples at 490 nm was recorded using a microplate reader

22   (SpectraMax M2, Molecular Devices). The absorbance of the cells incubated with only DMEM

1 was considered as 100 % of cell survival (Abs^{+ve}), and the cells treated by 0.5 % Triton X-100

2 was considered as 0 % (Abs^{-ve}). Cell survival was calculated using the following equation:

3   Cell survival (%) = [(Abs^{sample} – Abs^{-ve}) ÷ (Abs^{+ve} – Abs^{-ve})] × 100

4

5 ***Preliminary in vivo study in ectopic xenograft model***

6   The preliminary *in vivo* study was performed following the guidelines of the European

7 regulations. The experimental protocol was approved by the 'French Ministry of National

8 Education, Higher Education and Research': APAFIS#8293-2017032217458665v3. Seven

9 weeks old female NMRI nude mice were collected from Janvier Labs (France). The animals

10 were kept in the animal facility for one week for acclimatization and were given sufficient food

11 and water throughout the study.

12   After acclimatization period, the animals were anesthetized by temporary exposure to 2 %

13 isoflurane in oxygen to induce anesthesia followed by 1.5 % isoflurane in oxygen delivered by

14 face mask to maintain it. The U87MG cells were trypsinized and washed three times before

15 injected subcutaneously in the right flank of the mice (2 × 10^6 cells in 50 µL PBS). When the

16 tumor became palpable, tumor volume was measured using an electronic caliper using the

17 following equation: Volume = π/6 × length × width^2. Seven days after cell injection, the mice

18 were divided into 5 groups to have similar average tumor volume. The animals were anesthetized

19 (by above mentioned method) and received the following treatments by injections in the tail vein

20 on day 7 and day 10- Group 1: 70 µL Saline (n = 7); Group 2: 70 µL of LNC-blank equivalent to

21 822.4 mg LNC per kg of body weight (n = 8); Group 3: 70 µL of LNC-blank-fluoNFL2

22 equivalent to 822.4 mg LNC and 21.5 mg peptide per kg of body weight (n = 8), Group 4: 70 µL

23 of LNC-FcTriOH equivalent to 20 mg FcTriOH per kg of body weight (equivalent to 822.4 mg

1   LNC per kg of body weight) (n = 8); Group 5: 70 μL of LNC-FcTriOH-fluoNFL2 equivalent to

2   20 mg FcTriOH per kg of body weight (equivalent to 822.4 mg LNC and 21.5 mg peptide per kg

3   of body weight) (n = 8). The length and width of the tumor was followed regularly (every day in

4   the first week of treatment and then 3-times a week). Weight and behavior of the animals were

5   daily followed.

6

7   *Statistical analysis*

8   The experiments were performed at least 3 times. Results obtained from the experiments were

9   analyzed statistically using GraphPad Prism® software. Mean and standard deviation (SD) were

10   determined and values are represented as Mean ± SD. T-test or One way analysis of variance

11   (ANOVA) (with Tukey post-test to compare among individual groups, and Dunnett's post-test to

12   compare with control) was performed in the respective fields. P-value less than 0.05 (p <0.05)

13   was considered to be statistically significant.

14

15   **CONCLUSIONS**

16   In this study, we have shown that surface-functionalization with NFL peptide can enhance the

17   uptake of LNCs in human GBM cells in a dose-dependent manner. Moreover, the peptide-

18   functionalized LNCs reached the cytoplasm at much higher concentration compared to the non-

19   functionalized control LNCs. Additionally, the peptide functionalized LNCs were preferentially

20   internalized into GBM cells compared to healthy human astrocytes showing the targeting

21   capacity of the nanovector. The internalization of this nanocarrier in the U87MG cells was

22   energy-dependent and occurred by a combination of macropinocytosis, clathrin-mediated and

23   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 IC_{50}. 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 BBTB [48]. 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.

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1 *Supporting Information*

2 Electronic Supplementary Information (ESI) available: Figures showing the results of MTS

3 assays of blank LNCs and fluoNFL solutions. See DOI: 10.1039/x0xx00000x

4

5 *List of abbreviations*

6 BBB: blood-brain barrier; BBTB: blood-brain tumor barrier; CP: chlorpromazine; DAM: 5-

7 (N,N-dimethyl) amiloride hydrochloride; DiA: 4-(4-(dihexadecylamino)styryl)-N-

8 methylpyridinium iodide); DLS: dynamic light scattering; EE: Encapsulation efficiency; EPR:

9 enhanced permeability and retention; FcTriOH: 4-ferrocenyl-5,5-bis(4-hydroxyphenyl)-pent-4-

10 en-1-ol; fluoNFL: FAM-labelled NFL-TBS.40-63 peptide; GBM: glioblastoma; HPLC: high

11 performance liquid chromatography; IC50: inhibitory concentration 50; LNC: lipid nanocapsule;

12 MβCD: Methyl-β-cyclodextrin; NDDS: nanosized-drug delivery system; NFL: neurofilament

13 light subunit derived 24 amino acid tubulin binding site peptide NFL-TBS.40-63; NHA: normal

14 human astrocytes; NHS: normal human serum; NTA, nanoparticle tracking analysis; PDI:

15 polydispersity index; phalloidin-TRITC: phalloidin–tetramethylrhodamine-B-isothiocyanate;

16 PMA: phorbol-12-myristate-13- acetate; UPW: ultra-pure water.

17

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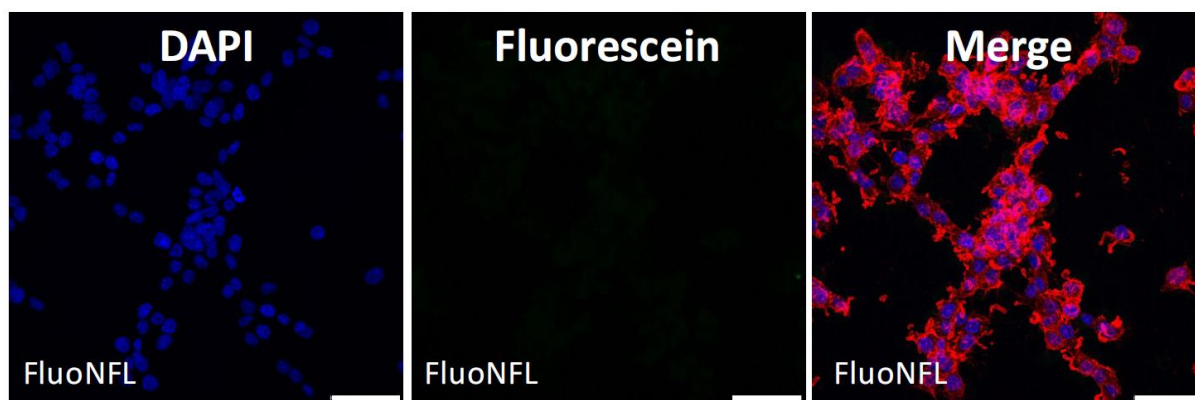1

2 # Supporting Information

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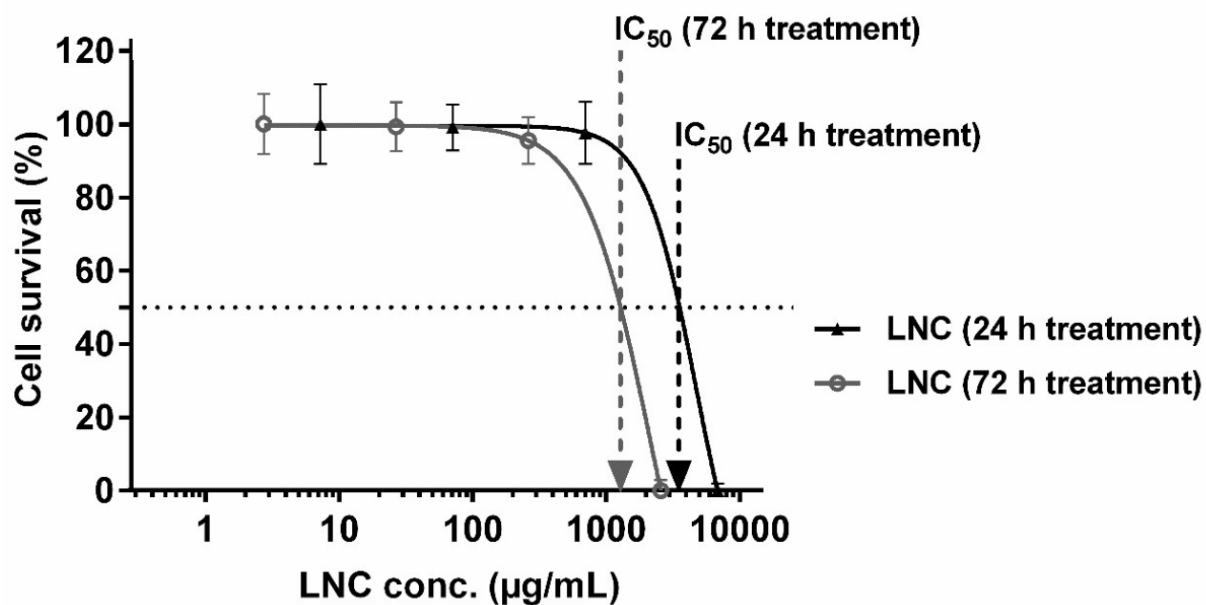
7 **Figure S1.** Survival percentages of U87MG cells after 24 h and 72 h treatment with

8 various concentrations of blank LNCs.

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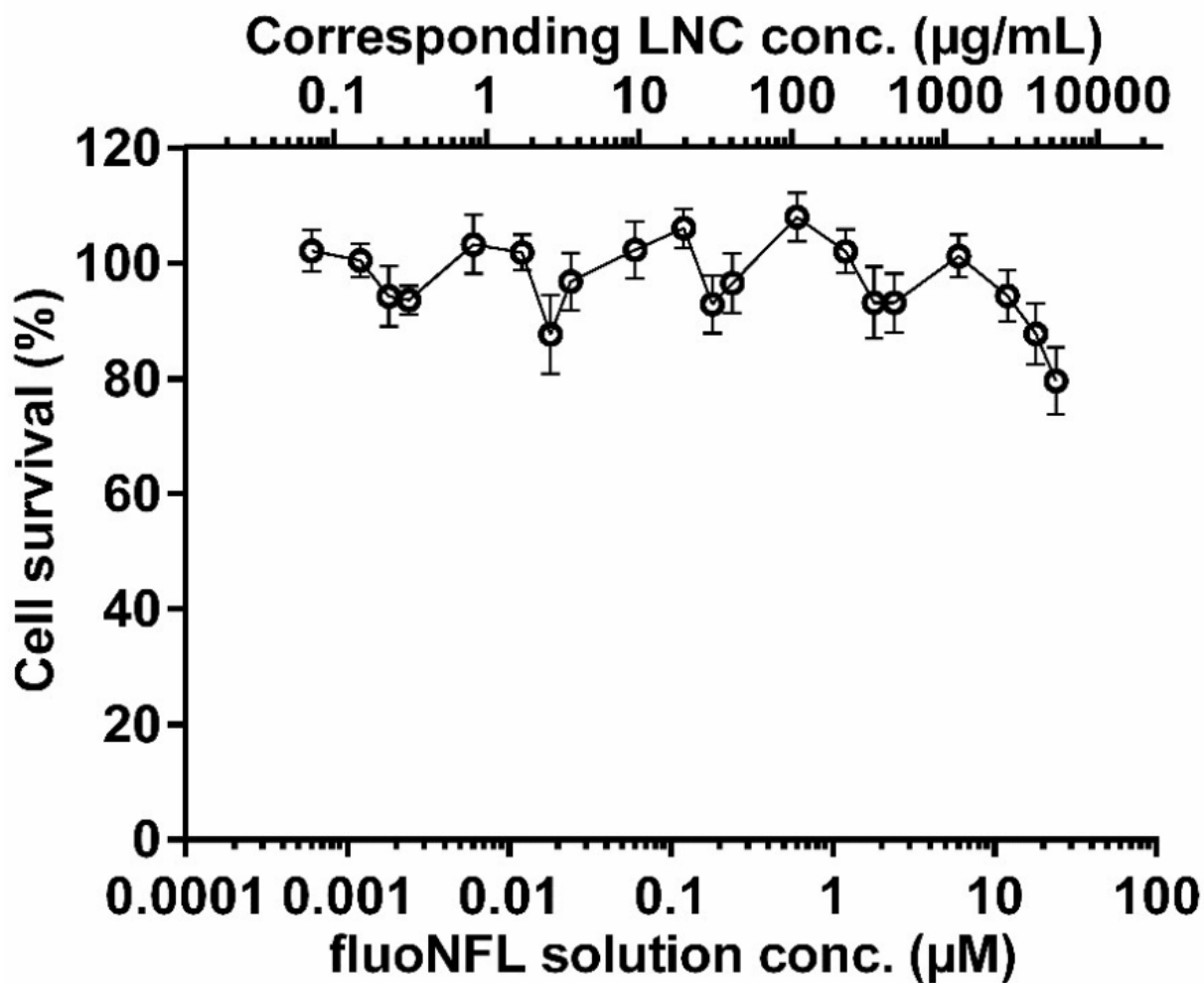
Figure S2. Survival percentages of U87MG cells after 72 h treatment with various
concentrations fluoNFL solution.

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

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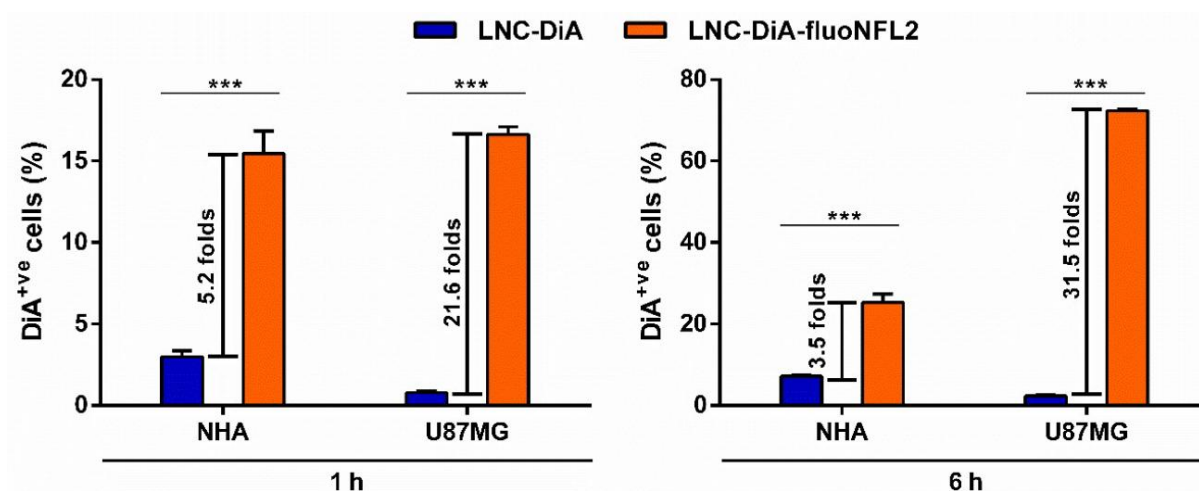
9 **Figure S4:** Enhanced LNC Internalization into NHA and U87MG cells due to LNC
10 surface functionalization using fluoNFL peptide. The cells were incubated with 1.23
11 mg/mL of LNCDiA and LNC-DiA-fluoNFL2 for 1 h and 6 h. Twenty thousand events per
12 sample were analyzed and percentages of DiA^{+ve}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

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