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

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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 a lipid nanocapsule (LNC) was studied to enhance nanovector internalization into human GBM cells. 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 the 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 and clathrin-mediated and caveolin-mediated endocytosis. A new ferrocifen-type molecule (FcTriOH), as a 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 the LNC surface is a promising approach for increased and preferential nanocarrier internalization into human GBM cells, and the FcTriOH-loaded LNCs are a promising therapy approach for GBM.

Introduction

Glioblastoma multiforme (GBM) is one of the most prevalent and fatal primary brain tumors classified by 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 past few decades, the median survival only altered from 8.3 to 14.6 months over the past 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 the enhanced permeability and retention (EPR) effect, and improve survival time of animals.6 Among various nanocarriers, lipid nanocapsules (LNCs) have been reported in numerous studies as promising NDDS for carrying hydrophobic drugs due to their characteristic oily
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 purposes. LNCs were evaluated and showed promising in vitro and in vivo results against GBM in numerous studies. 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. FcTriOH belongs to the newer hydroxypropyl series of ferrocifens that can convert into novel tetrahydrofuran-substituted quinone methide. Compared to the corresponding ferrocifen that can convert into novel tetrahydrofuran-substituted quinone methide (by cyclization of the hydroxyalkyl chain). In this study, the intrinsic 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. 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. This peptide was evaluated as a potential GBM-targeting moiety on the LNC surface in rat or mouse GBM cell lines. 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 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, the 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. The 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. The 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.

**Results**

**Physicochemical characteristics of the nanocapsules**

LNCs were prepared by a phase-inversion technique as previously described. 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. The initial molar concentration of the NFL peptide for the LNC-fluoNFL1 was kept the same as in previously published studies (1 mM) whereas it was 3-fold higher in LNC-fluoNFL2. Final concentrations of the adsorbed fluoNFL (％ w/w), determined by HPLC, on LNC-fluoNFL1 and LNC-fluoNFL2, were 0.40 ± 0.01% and 2.49 ± 0.01%, respectively.

Particle size, the 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

![Fig. 1 Structure of FcTriOH: 4-ferrocenyl-5,5-bis(4-hydroxyphenyl)-pent-4-en-1-ol.](image)

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<th>Table 1 Physicochemical characteristics of the nanocapsules</th>
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<td><strong>Formulation</strong></td>
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<tr>
<td>Control LNC</td>
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One-way ANOVA with Dunnett’s post-test. p < 0.05 is denoted by (*), p < 0.01 by (**) and p < 0.001 by (***) as compared to control LNC.
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 were not altered after FcTriOH encapsulation with/without fluoNFL functionalization compared to respective unloaded LNCs.

**Interaction between LNCs and fluoNFL**

The interaction between the LNC surface and NFL peptide in a formulation equivalent to LNC-fluoNFL1 has been 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 the NaCl concentration was increased up to 1 mM. However, as the 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.

Moreover, LNC-fluoNFL2 was dialyzed against 0.05 M Tris buffer at 37 °C and 75 rpm using a dialysis bag having MWCO 100 kDa. 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.

**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 the surface area of the LNC formulations. The percentage of CH50 unit consumptions by the control LNCs, and the peptide functionalized LNCs were plotted against the surface area of the nanocapsules in 1 mL of NHS (Fig. 4). The complement consumption by all three nanocapsules increased as the 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 cm\(^2\) mL\(^{-1}\) NHS. The complement consumption by LNC-fluoNFL2 was slightly higher and reached 21.0% at the same surface area.

![Fig. 2](image1.png) 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 (***)). \( n = 3 \).

![Fig. 3](image2.png) FluoNFL desorption kinetics from the LNC-fluoNFL2 surface in 0.05 M Tris buffer pH 7.4 at 37 °C and 75 rpm.

![Fig. 4](image3.png) Complement consumption at 37 °C by control LNCs, LNC-fluoNFL1 and LNC-fluoNFL2.
Effect of surface-functionalizing fluoNFL concentration on LNC internalization into human GBM cells

The LNC concentration used for the cellular uptake studies was 1.23 mg mL⁻¹ (corresponds to 11.1 μM NFL in LNC-fluoNFL2), which was primarily selected based on previously described safe concentrations of LNCs and NFL peptide.¹⁹,²²,²³ Moreover, results of the MTS assays on U87MG cells treated with the peptide solutions or blank LNCs showed that cell viability did not decrease at these concentrations (ESI: Fig. S1 and S2). The U87MG cells were treated with DiA-labelled LNCs (LNC-DiA, LNC-DiA-fluoNFL1 and LNC-DiA-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 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.

Moreover, to investigate the necessity of peptide adsorption on LNC (during the formulation of LNC-fluoNFL2) to enhance its cellular uptake, DiA-labelled LNC and fluoNFL (at the 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).

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

To visualize whether the LNCs were on the cell surface or inside the cytoplasm, orthogonal sections of the stacked images were analyzed (Fig. 8). Indeed, nearly all the fluorescence was observed 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.

**Preferred 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, internalizations of DiA loaded LNC and LNC-fluoNFL2 into NHA were measured and compared with 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-fold and 3.5-fold 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-fold and 31.5-fold at 1 h and 6 h, 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.

**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 under different energetic conditions i.e. at 4 °C and ATP-depleted conditions for 1 h and 6 h (Fig. 10a).
Fig. 7 Representative confocal microscopy images of enhanced LNC internalization into U87MG cells due to the LNC surface-functionalizing fluoNFL peptide. Cells were treated at 37 °C for 6 h with 1.23 mg mL\(^{-1}\) of LNC-DiA and LNC-DiA-fluoNFL2. Blue is DAPI staining (nuclei), green is DiA staining (LNC) and red is phalloidin-TRITC staining (F-actin, cytoskeleton). White bar = 20 μm. ESI: Fig. S3.†

Fig. 8 Orthogonal sections of stacked confocal microscopy images showing LNC internalization into U87MG cells. The majority of LNC-DiA-NFL2 was localized into the cell cytoplasm, whereas LNC-DiA was chiefly localized on the cell surface. The cells were treated at 37 °C for 6 h with 1.23 mg mL\(^{-1}\) of LNC-DiA and LNC-DiA-NFL2. Blue is DAPI staining (nuclei), green is DiA staining (LNC) and red is phalloidin-TRITC staining (F-actin, cytoskeleton). White bar = 10 μm.
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, the 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 under 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 under an ATP-depleted condition at 1 h compared to n.c. (Fig. 10b). Therefore, LNC-fluoNFL2 uptake in U87MG cells was a temperature and energy-dependent process, similarly to fluoNFL as previously reported.18

To further evaluate the possible uptake pathway(s) of the peptide-functionalized LNCs, exclusion of particular endocytosis mechanisms was achieved using inhibitors of the foremost endocytosis pathways. The cells were pretreated for 30 minutes with different inhibitors followed by 1 h treatment with LNC-DiA-NFL2. LNC uptake was significantly inhibited in the presence of each of these inhibitors (Fig. 11a). LNC-DiA-NFL2 internalization was the lowest in the presence of DAM, followed by CP, MβCD 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 the presence of CP and MβCD, and lastly PMA.

**In vitro efficacy on U87MG cells**

To evaluate the *in vitro* efficacy of FcTriOH loaded LNC formulations, cell viability was evaluated by the MTS assay after 72 h of treatment with the formulations (Fig. 12). The IC$_{50}$ of FcTriOH solution was 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-fold and 2.3-fold 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$^{-1}$) compared to the drug-loaded LNCs (ESI: Fig. S1†).
not show any toxicity in the tested concentrations (ESI: Fig. S2†).

**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 the 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 the saline treated group, the relative tumor growth for the 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.

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

Covalent coupling of the peptide on the distal end of PEG chains was not performed as it diminished the GBM targeting capability of NFL. Therefore, surface-functionalization of LNCs was performed by simply adsorbing different amounts of the peptide onto the LNC surface over a 24 h period. As size, zeta potential and surface coating can profoundly impact the in vivo fate of the nanovectors, these properties of the developed LNCs were characterized (Table 1). The particle size of the control LNCs was 57 ± 2 nm, whereas the diameters of LNC-fluoNFL1 (NFL 1 mM) and LNC-fluoNFL2 (NFL 3 mM) were about 4 nm and 7 nm larger, respectively, signifying a potential higher amount of fluoNFL adsorbed onto the surface. Similarly, peptide adsorption increased the zeta potential of LNC-fluoNFL1 and LNC-fluoNFL2 by +2 and +7 mV, respectively, compared to control LNC. This variation of surface charge can be explained by the net positive charge of the NFL peptide at physiological pH. The changes in size and zeta potential for LNC-fluoNFL1 were similar to the one reported by Carradori et al. Moreover, the LNC size after fluoNFL adsorption was well below 100 nm, which can be beneficial for diffusion in the cerebral extracellular space. The PDI of all three formulations was less than 0.2; therefore they can be considered as monodispersed. The concentration of LNC-adsorbed fluoNFL was quantified indirectly by measuring the free peptide concentration after separating them using centrifugal filters with an MWCO of 100 kDa. The concentration of LNC-adsorbed peptide in LNC-fluoNFL1 was 0.40% w/w, which was in correspondence with the concentration reported by Balzeau et al. and Carradori et al. The concentration of LNC adsorbed fluoNFL in LNC-fluoNFL2 was 2.49% (w/w), which was about 6-fold higher compared to LNC-fluoNFL1 although the concentration of peptide initially added was only 3-fold higher. The number of peptide molecules per LNC particle can be calculated from the particle concentration obtained by NTA and the adsorbed NFL concentration quantified by HPLC. About 240 and 1530 peptides were adsorbed per LNC particle in LNC-fluoNFL1 and LNC-fluoNFL2, respectively. Torchilin et al. determined the number of TAT peptides on 200 nm liposomes by radiolabeling the peptide and measuring liposome-associated radioactivity. About 500 peptides per liposome was calculated, which was included in the range of fluoNFL chains used in our formulations. About 0.016 and 0.102 fluoNFL molecules will be present per nm² surface area in LNC-fluoNFL1 and LNC-fluoNFL2, respectively.

Fig. 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 × 10⁶ cells (in 50 μL 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 were followed regularly. Statistical analysis was performed with one-way ANOVA with the 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).
dispersion of LNCs became a gel after adsorption by an initial concentration of 4 mM fluoNFL (therefore, NFL concentrations above 3 mM were not tested). This also indicated that the peptide–LNC mixture might start to form a rigid network at high peptide concentrations. Self-assembly of peptides has been described to form hydrogels in the literature. Alteration in environmental conditions (e.g. pH and ionic strength) can trigger interaction among peptide chains resulting in physical cross-linking and filament growth to form viscoelastic solids. Addition of LNC dispersion may alter such environmental conditions of the peptide solution and result in formation of semi-solids.

Balzeau et al. has reported that NFL interacts with the polar PEG chains of Kolliphor whereas Carradori et al. suggested that the interaction was possibly by a combination of electrostatic forces and other weak forces i.e. van der Waal’s forces and hydrophobic forces. We evaluated the effect of NaCl and Tris buffer concentration on LNC-fluoNFL2 size by incubation with different concentrations of these solutions and subsequently measuring their diameter in DLS (Fig. 2). Contrasting to previous studies, the significant difference of nanocapsule diameter compared to control LNCs was maintained nearly throughout the NaCl concentration range. However, Tris buffer impacted more the size of LNC-fluoNFL2 compared to NaCl, and no significant difference of particle size was observed above 0.05 M concentration. It can be hypothesized that the possible self-entanglement of the peptide in LNC-fluoNFL2 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 whether fluoNFL will be rapidly removed from the LNC surface after dilution, LNC-fluoNFL2 was dialyzed (MWCO 100 kDa dialysis bag) against 1× 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 the LNC surface was slow and gradual and only 33.6% peptide was desorbed after 6 h (Fig. 3). Moreover, NFL-functionalized LNCs were reported to maintain their characteristics in cell culture medium. 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 (the 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 were 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). CH50 unit consumption by LNC-fluoNFL1 was similar to what was shown by the control LNCs (Fig. 4). However, CH50 unit consumption by LNC-fluoNFL2 was slightly enhanced compared to the other two formulations. This can be attributed to the increased size as surface area recognition by the complement is proportional to the particle diameter, or to the altered zeta potential. Overall, the complement consumption by all three formulations was low, even at high surface area (calculated by NTA) in theory, formulations should not be quickly removed from the bloodstream by MPS.

Previously, Balzeau et al. showed that the internalization of LNC in mouse GBM cells can be enhanced by adsorbing the NFL peptide on its surface. However, cellular uptake on nanocarriers can be cell specific as the interacting plasma membrane composition (i.e. ligands, receptors and endocytosis apparatus) varies among cell lines. Therefore, as a potential therapeutic strategy for human disease, it was necessary to characterize the internalization kinetics of the LNC with/without the surface-adsorbed NFL peptide in a human GBM cell line at a non-toxic concentration. Moreover, Lépinoux-Chambaud et al. reported that the extent and pathway of NFL internalization into U87MG cells were dependent on the extracellular peptide concentration. Therefore, the effect of LNC surface-functionializing fluoNFL peptide concentration on LNC internalization by U87MG human GBM cells was evaluated in this study. For this purpose, LNCs were fluorescently labelled by encapsulation of a hydrophobic dye, DiA, and their cellular uptake was quantified using fluorescence-activated cell sorting (FACS). The LNC concentration used for the cellular uptake studies was 1.23 mg mL−1, having 11.1 μM of NFL-peptide in LNC-fluoNFL2. The blank LNCs and the peptide solutions were non-toxic at these concentrations as observed in the MTS assays (ESI: Fig. S1 and S2f). To identify and separate dead cells, the FACS samples were suspended in 0.12% w/v of trypan blue for measurements in a flow cytometer and signals in a 655 nm long-pass filter was detected. Trypan blue can enter inside cells with damaged membranes, complex with proteins and emit fluorescence around 660 nm that can be detected in FACS. However, a maximum of 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 the 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 uptake of the 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) on the nanocarrier surface can have a significant impact on the rate and the extent of the nanovector cellular internalization in human GBM cells. Therefore, this strategy could be used to improve nanocarrier targeting efficiency to other types of cells in which the peptide can efficiently enter i.e. brain neural stem cells.\(^{18,22,41}\)

Previously, Paillard \textit{et al.} reported that the internalization of untargeted LNCs was not preferentially targeted into GBM cells and entered also healthy astrocytes.\(^{38}\) Additionally, surface-functionualization 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 the presence of various endocytosis pathway inhibitors. The internalization of the nanocarrier was significantly reduced (compared to 37 °C) when incubated under different energetic conditions (4 °C and ATP-depleted condition) (Fig. 10a). Under ATP-depleted conditions, no LNC-DiA-fluoNFL2 uptake was observed after 1 h, but an increase to about 25% of the n.c. after 6 h was observed. In contrast, 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 6 h, these nanoobjects 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 cells was an energy-dependent active process. A comparable trend was observed in cellular uptake of fluoNFL alone (Fig. 10b), which was also mentioned in previous reports.\(^{18}\)

The dependency of cellular uptake on energy indicates that the internalization possibly occurs by endocytosis. To further illustrate the particular internalization pathway(s) involved, the cells were pretreated with various inhibitors of the chief endocytosis pathways. Treatment with MβCD 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 peptide\(^{23}\) (also observed in our experiments, Fig. 11b), the internalization of 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 and 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 the 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 has shown significantly enhanced activity on hormone-independent breast cancer cells compared to the acyclic ferrocphenol.\(^{12}\) This was the first time this compound was studied \textit{in vivo} on a human GBM tumor model in mice. The \textit{in vitro} antiproliferative activity of FcTriOH was assessed by the MTS assay. The cells were treated with 0.1–100 μM of FcTriOH and its formulations for 72 h. Up to 0.1 μM, cell survival was above 80% for all treatment groups (Fig. 12). Between 0.1 and 10 μM, the cell survival percentage drastically reduced for cells treated with FcTriOH, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2 resulting in IC\(_{50}\) values of 1.31 μM, 1.05 μM and 0.46 μM, respectively. The survival of cells treated with control LNC reduced significantly between 10 and 100 μM with an IC\(_{50}\) of 22.5 μM. Corresponding concentrations of fluoNFL solution did not alter cell viability, which was also reported previously.\(^{18}\)

In a preliminary \textit{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\(^{-1}\) FcTriOH. As no previous reports about this FcTriOH administration in animals were available, the dose was chosen based on previous \textit{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 by day 17 (Fig. 13) which was maintained up to day 22. The mice 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 possibility occurs as the drug is eliminated resulting in its antiproliferative effect fading and the tumor growing back. In fact, several preclinical studies have used a much higher number (6 to 20) of i.v. injections and observed a significant difference in 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 compared to saline treated groups, the differences 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 future studies.

The NFL-peptide concentration of the 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 their 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 in 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 by Lipoid GmbH (Germany), and caprylic/capric triglycerides (Labrafac Lipophile WL1349) were supplied by Galtesfosse (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⁻¹ l-glucose (DMEM) were provided by Lonza (France). Methyl-β-cyclodextrin (MβCD), 5-(N,N-dimethyl) amiloride hydrochloride (DAM), chlorpromazine (CP), phalloidin-tetramethyl-rhodamine-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)styril)-N-methylpyridinium iodide (DiA), 4',6-diamidino-2-phenylindole (DAPI), Trypsin-EDTA 1×, non-essential amino acids solution 100× (NEAA), fetal bovine serum (FBS) and ProLong Gold antifade were collected from Thermo Fisher Scientific (USA). 3-Carboxymethoxyphenyl-2-(4-sulphophenyl)-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 the phase inversion temperature technique. 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 ref. 19 and 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 were also functionalized with fluoNFL by the same method as LNC-fluoNFL1 and LNC-fluoNFL2.
Preparation of FcTriOH-loaded lipid nanocapsules. FcTriOH-loaded LNC (LNC-FcTriOH) was prepared similar to control LNCs, except that 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 the method identical to 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 a previously published article.24 The 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 the dynamic light scattering (DLS) technique using a Zetasizer Nano ZS system (Malvern Instruments Ltd, UK). The LNCs were diluted 100-fold in UPW before the analysis. The measurements were performed at a backscatter angle of 173°. The measured average values were calculated from 3 runs, with 10 measurements within each run.

The zeta potential of the nanocarriers was measured using laser Doppler micro-electrophoresis using a Zetasizer Nano ZS system (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 a NanoSight NS300 system (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 a 30 µL min⁻¹ rate. A 405 nm laser was used to illuminate the particles, and their Brownian motion was recorded in three 60 s videos (25 fps) using the sCMOS type camera of the instrument. Subsequently, the NTA software (NTA 3.2 Dev Build 3.2.16) analyzed the recordings, tracked the motion of the particles and calculated the number of particles in the samples. The experiment was performed in triplicate.

High-performance liquid chromatography. The peptide concentration was indirectly measured by quantifying the free peptide present in the formulations using a supplier recommended HPLC method. Briefly, the fluoNFL functionalized LNCs were filtered by centrifugation at 4000g for 30 min using Amicon Ultra-0.5 mL centrifugal filters having a molecular weight cut off (MWCO) of 100 kDa (Millipore). The filtrate containing the free fluoNFL was collected and the peptide dosage was performed in a HPLC system (Waters, France). A C18 analytical column (250 × 4.6 mm, 5 µm, Waters, France) was used at room temperature. 0.1% TFA in UPW and 0.1% TFA in acetonitrile were used as mobile phases (gradient: 80 : 20 → 55 : 25, 25 min). The flow rate was 1 mL min⁻¹, injection volume was 10 µL and fluoNFL was quantified by a UV detector at λ of 220 nm. Analysis of the data was performed by Empower 3 software (Waters). The retention time was 18 min. Calibration curves were established by quantifying the area under the curves (AUCs) of 1–100 µg mL⁻¹ solutions of fluoNFL in UPW. The peptide solution and LNCs alone were also filtered and quantified as positive and negative controls.

To quantify total (encapsulated and unencapsulated) drug concentration, LNCs were broken by mixing vigorously with an appropriate volume of ethanol (40 fold for LNCs prepared for in vitro experiments, 100-fold for concentrated LNCs prepared for in vivo experiments) to keep the dissolved drug concentration between 5 and 75 µg mL⁻¹. To quantify the unencapsulated drug concentration, formulations were placed on centrifugal concentrator devices with a polyethersulfone membrane (MWCO 30 kDa, Amicon Ultra-500, Millipore) and centrifuged at 4000g 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 determined in a HPLC system (Waters, France). A C18 analytical column (250 × 4.6 mm, 5 µm, Waters, France) was used at room temperature. UPW and acetonitrile (45 : 55, v/v) were used as mobile phases. The flow rate was 1 mL min⁻¹, injection volume was 10 µL and FcTriOH was detected at 304 nm. Analysis of the data was performed by Empower 3 software (Waters). The retention time of FcTriOH was 8.1 min.

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

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

Drug loading was calculated using the following equation:

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

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, and 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 kDa, Spectra/Por® biotech grade cellulose ester membrane, SpectrumLabs, Netherlands), dialyzed against Tris buffer (0.05 M, pH 7.4) at 37 °C, and 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 the 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. In brief, aliquots of NHS were incubated with increasing concentrations of the LNCs at 37 °C for 1 h. Subsequently, 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. Particle number in the LNC dispersion was determined by NTA and nanocarrier concentration per mL of NHS was calculated according to the following equation:

$$\text{Particle number per mL of NHS} = \frac{\text{Particle conc. in NDDS dispersion} \times \frac{\text{vol. of NDDS added}}{\text{vol. of NHS}}}{10^6}$$

Subsequently, the 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 at 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 x 10^5 cells per well concentration for 24 hours. Subsequently, they were treated with the different DiA-labelled LNCs (1.23 mg mL^-1) for 0.5, 1, 6 and 24 h. Afterwards, the cells were washed three times with ice-cold phosphate buffer saline 1× (PBS), and detached by incubating 5-10 minutes with Trypsin-EDTA 1×. 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% v/v) and the percentage of DiA positive (DiA™) or FAM positive (FAM™) cells were analyzed by a 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 (the method mentioned above) at 37 °C and the percentage of DiA™ cells was measured using the above-mentioned method, and compared with the results of U87MG cells.

Mechanism of fluoNFL-functionalized LNC internalization in U87MG cells. To evaluate the dependency ofNFL-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 (NaF 10 mM and 2-deoxy-D-glucose 6 mM) to deplete cellular ATP. Subsequently, the cells were treated for 1 and 6 h with the LNC-DiA-fluoNFL2 and percentage of DiA™ 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^-1, DAM 1 mM, CP 50 μM and PMA 10 μg mL^-1) for 30 min at 37 °C (ref. 23) followed by 1 h treatment with the nanocarrier and the percentage of DiA™ 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™ cells to assess whether 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 x 10^4 cells per well) in 24 well plates containing coverslips and incubated at 37 °C for 72 h (the 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^-1 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 μg mL^-1 of phallolidin-TRITC for 1 h at room temperature. Subsequently, the cells were washed twice with PBS and incubated with 3 μM DAPI for 10 minutes. Finally, the cells were washed 3-times with PBS and the coverslips were mounted using the 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 405 nm wavelength and recorded at 409-453 nm (blue channel), DiA and fluoNFL were excited with 548 nm wavelength and recorded at 558-666 nm (green channel) whereas TRITC was excited with 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 the MTS assay. In brief, the
U87MG cells were seeded in 96 well plates (5000 cells per well) and incubated for 24 h. Then the medium was replaced with various concentrations of LNCs (60–600 000 fold diluted in DMEM) (LNC-blank, LNC-FcTriOH and LNC-FcTriOH-fluoNFL2), FcTriOH (22–220 000 fold dilution in DMEM) and fluoNFL (125–4 983 383 fold dilution in DMEM) and treated for 72 hours at 37 °C. After that, the content of each well was replaced with 100 μL of fresh DMEM. Additionally, 20 μL 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 were considered as 0% (Abs\(^{ve}\)). Cell survival was calculated using the following equation:

\[
\text{Cell survival} \, (\%) = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{ve}})}{(\text{Abs}_{\text{ve}} - \text{Abs}_{\text{ve}})} \times 100
\]

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 week old female NMRI nude mice were obtained from Janvier Labs (France). The animals were kept in the animal facility for one week for aclimatization and were given sufficient food and water throughout the study.

After the aclimatization 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 a face mask to maintain it. The U87MG cells were trypsinized and washed three times before being injected subcutaneously in the right flank of the mice (2 × 10⁶ cells in 50 μL PBS). When the tumor became palpable, tumor volume was measured using an electronic caliper using the following equation:

\[
\text{Volume} = \frac{\pi \times \text{length} \times \text{width}^2}{\text{height}}
\]

Seven days after cell injection, the mice were divided into 5 groups having similar average tumor volume. The animals were anesthetized (by the above mentioned method) and received the following treatments by injections in the tail vein on day 7 and day 10:

Group 1: 70 μL saline (n = 7); Group 2: 70 μL of LNC-blank equivalent to 822.4 mg LNC per kg of body weight (n = 8); Group 3: 70 μL 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 μL 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); and Group 5: 70 μL 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 were followed regularly (every day in the first week of treatment and then 3-times a week). The weight and behavior of the animals were daily followed.

Statistical analysis. The experiments were performed at least 3 times. The 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 ± SD. T-Test or one way analysis of variance (ANOVA) (with the 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 concentrations 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 and clathrin-mediated and caveolin-mediated endocytosis, a pathway similar to 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 its 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. Overall, enhancement of NFL peptide concentration on the LNC surface is a promising strategy for greater and targeted nanocarrier internalization into human glioblastoma cells, and the FcTriOH-loaded LNCs are a promising therapy approach for glioblastoma.

Conflicts of interest

There are no conflicts to declare.

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}steryl)-N-methyl-pyridinium iodide
- DLS: Dynamic light scattering
- EE: Encapsulation efficiency
- EPR: Enhanced permeability and retention
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