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Investigating the functionalization of liposomes with NFL-TBS. 40-63 peptide as a promising drug delivery system

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ABSTRACT

The NFL-peptide was discovered almost 20 years ago, and its targeting properties were assessed alone or in combination with lipid nanocapsules (LNC), magnetic porous silicon nanorods, or gold nanoparticles. Results highlighted a better targeting of cancer cells, in particular glioblastoma and pancreas cancer. Considering the large use of liposomes (LPs) as an hydrophilic drug delivery system, this study explored the possibility to functionalize liposomes with three different sequences of NFL-peptides: native (NFL-peptide), biotinylated (BIOT-NFL) and coupled to fluorescein (FAM-NFL). Dynamic Light Scattering (DLS) complemented by cryo-electron microscopy (CEM) showed a peculiar ultrastructural arrangement between NFL-peptides and liposomes. Based on this architectural interaction, we investigated the biological contribution of these peptides in LPs-DiD glioblastoma cellular uptake. Flow cytometry complemented by confocal microscopy experiments demonstrated a consequent and systematic increased uptake of LPs-DiD into F98 cells when their surface was decorated with NFL-peptides. The intra-cellular distribution of these liposomes via an organelle tracker indicated the presence of LPs-DiD in lysosomes after 4 h. Based on the properties of this NFL-peptide, we showed in this work the crucial role of NFL peptide as an effective and promising actor to potentiate nanoparticles entry in glioblastoma cell lines.

1. Introduction

Glioblastoma multiforme (GBM) is the most lethal primary brain cancer characterized by a very poor median survival of less than 2 years (Batash et al., 2017). Surgical tumor resection followed by concomitant Temozolomide chemotherapy and radiotherapy remains the main conventional treatment for GBM patients (Stupp et al., 2005). Nevertheless, the highly proliferative and invasive behavior of the tumor induce frequent recurrences (Carlsson et al., 2014). Additionally, the drugs efficiency is limited by the presence of the blood brain barrier (BBB) and blood–brain tumor barrier (BBTB) which strongly restricted compounds pathway (Van Tellingen et al., 2015). Since several years, one of the essential key-point in GBM treatment is to specifically target tumor cells

and overcome BBB by developing an efficient curative system. In this context, the development of new drugs delivery system (DDS) targeting cancer cells is a promising therapeutic strategy.

Liposomes (LPs), discovered in 1963 by Alec Bangham, are spherical organic vesicles ranging from 100 to 500 nm in diameter, composed of a phospholipid bilayer and aqueous core (Bangham AD, 1965). Thanks to their polyvalent composition, LPs can hold a large spectrum of lipophilic and hydrophilic molecules (Zamani et al., 2018). Moreover, interest of using LPs has been based on their biocompatibility and biodegradability, low immunogenicity and cytotoxicity, high loading efficacy as well as their possibility to pass BBB. In clinical trials, liposomes are commonly used, and have already proven their benefits as nanocarriers through FDA approved examples. In 1995, the first drug called Doxil®,

Abbreviations: GBM, Glioblastoma multiforme; NFL-TBS.40–63 or NFL-peptide, Neuro Filament Low subunit Tubulin Binding Site; BIOT-NFL, Biotinylated NFL-peptide; FAM-NFL, 5-carboxyfluorescein NFL-peptide; LPs, Liposomes; DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate; LPs-DiD, Liposomes loaded with DiD; LPs-DiD-BIOT-NFL, Liposomes loaded with DiD functionalized with BIOT-NFL-peptide; LPs-DiD-FAM-NFL, Liposomes loaded with DiD functionalized with FAM-NFL-peptide; POPC, Phospholipid 1-palmitoyl-2-oléoyl-*sn*-glycéro-3-phosphocholine; CHOL, Cholesterol; CEM, Cryo-electron microscopy; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's Phosphate Buffered Saline; SEC/UPLC, Size Exclusion Chromatography/Ultra-Performance Liquid Chromatography.

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composed of pegylated liposomes encapsulating Doxorubicin was developed to target breast, lung and ovarian cancers. (Raj et al., 2021). More recently, the Vyxeos®, a liposomal formulation with Daunorubicin and Cytarabine showed curative results against Acute Myeloid leukemia (Krauss et al., 2019). These last examples properly highlight the increase of drug's efficiency when they were encapsulated in LPs. However, researchers have focused on improving these nano-drugs to target cancer cells more effectively. Thus, preclinical studies have successfully demonstrated the absorptive capacity of liposomes through surface modifications including antibodies, proteins and peptides.

In fact, peptides have already been used therapeutically to functionalize liposomes. Qin et al. demonstrated an important anti-proliferative activity with LPs-Paclitaxel decorated by the cyclic arginine-glycine-aspartic acid peptide (cRGD) against C6 glioma tumor and spheroids cells (Qin et al., 2014). Other studies have focused on the dual targeting properties of peptides to modify liposomes for GBM therapy. A known cell-penetrating peptide (CPP) associated to an homing peptide have been shown to promote the uptake of Doxorubicin-containing liposomes to induce tumor cell necrosis: TAT, derived from the human immunodeficiency virus (VIH) and T7 peptide which has an affinity for endocytic transferrin receptor (Tfr), a receptor overexpressed in BBB and GBM tumors (Zong et al., 2014).

In our laboratory, a peptide called NFL-TBS.40–63 was discovered and used as a promising candidate to target and destroy GBM. The activity of this peptide is based on its capacity to bind tubulin expressed in GBM cells and to inhibit its polymerization, which causes the cells death by apoptosis (Berges et al., 2012). Interestingly, this NFL peptide can enter massively in several types of cancer cells, including GBM cancer and stem cells, neuroblastoma cells (NGP), breast cancer cells (MCF7), colorectal cancer cells (LS187), and pancreatic cells and increase the internalization of nanoparticles (Balzeau et al., 2013; Berges et al., 2012; Bocquet et al., 2009; Griveau et al., 2022). Moreover, this peptide has been used as an active targeting molecule for lipid nanocapsule (LNC) charged in Paclitaxel (PTX) and functionalized by NFL, which significantly reduced the GBM tumor volume by 75–80 % in a GL261 mouse model (Balzeau et al., 2013).

Following several physicochemical investigations of this peptide it has been possible to highlight the importance of its sequence on the ultrastructural organization into alpha-helical or β -turn motifs and its auto-assembly into nanofilaments (Berges et al., 2012; Alnemeh-Al Ali et al., 2022). This unique structure–function improves the uptake of this peptide by cancer cells and increases the uptake of nanovectors to which they are attached, including lipid nanocapsules, gold nanoparticles, and magnetic porous silicon nanorods (Balzeau et al., 2013, Arib et al., 2022, Chaix et al., 2022, Griveau et al., 2022).

In this study, we evaluated the interaction of this peptide with liposomes to develop a new drug delivery system to target GBM tumor cells. Neutral liposomes were functionalized with NFL-peptide, biotinylated (BIOT-NFL-peptide) or coupled with 5-carboxyfluorescein (FAM-NFL-peptide), then they were characterized by Dynamic Light Scattering (DLS) and cryo-electron microscopy (CEM). In parallel, using an *in vitro* cellular model, we explored their internalization in F98 cells via flow cytometry (FACS) as well as confocal microscopy analysis, and possible toxicity by mitochondrial activity quantification (Resazurin). This uptake study was complemented by an examination of the cellular biodistribution of liposomes through live confocal analysis. Taken together, the results indicate that liposomes can be functionalized with this NFL peptide, and that this significantly increases the entry of liposomes into GBM cells. Interestingly, the relationship between the ultrastructure and cellular uptake capacity varies significantly according to the types of modifications of each NFL peptide (alone or with Biotin or FAM).

2. Material and methods

2.1. Reagents

The distinct type of NFL-TBS.40–63 peptide (NH₂-YSSY-SAPVSSSLSVRRSYSSSSGS-CONH₂) (NFL-peptide), biotinylated (BIOT-NFL-peptide), and 5-carboxyfluorescein (5-FAM-NFL-peptide) were synthesized by PolyPeptide Group (Strasbourg, France). Deionized water was provided from Merck-Millipore (Darmstadt, Germany) via a MilliQ plus system.

2.2. Peptide preparation

For all experiments, the distinct NFL-TBS.40–63 peptides (NFL-peptide, BIOT-NFL, and FAM-NFL) were dissolved in sterile water at the initial concentration of 1 mmol/L. At this concentration, the BIOT-NFL-peptide can spontaneously form filaments.

2.3. Thin-film hydration liposomes formulation and functionalization with NFL-peptides

Small unilamellar liposomes were formulated according to the usual thin-film hydration method (Thabet et al., 2022). Briefly, a solution of the synthetic phospholipid 1-palmitoyl-2-oléoyl-*sn*-glycéro-3-phosphocholine (POPC, Avanti Polar Lipids, Inc, Alabaster, AL, USA); Cholesterol (CHOL, Sigma-Aldrich 36, 279–4); DiD solution (1,1-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate, D7757; Thermo Fisher) respectively in 4:4.5:1 M ratio was prepared in chloroform. A lipid film was produced in a round-bottom flask through rotary evaporation and dried overnight under vacuum. Then, the film was hydrated in a NaCl buffer (pH 7.4, 0.1 M) for 1 h at 60 °C. Multilamellar liposomes were obtained after five cycles of heat in a 60 °C water bath and freeze with liquid nitrogen at –80 °C. After cycles, unilamellar LPs were obtained according two methods: on one side, by probe sonication (Misonix) for 2 x 6 min at a frequency of 5 kHz and then 10 kHz, and secondly by extrusion through 100 nm polycarbonate membrane (Avanti Polar Lipids, Inc, Alabaster, AL, USA). Experiments were conducted at least in triplicate.

For targeting purposes, POPC-CHOL-LPs were surface-decorated by NFL-peptide, BIOT-NFL-peptide, or FAM-NFL-peptide. To achieve this, liposomal formulations with or without DiD dye were gently stirred with NFL-peptides overnight at room temperature to promote peptide adsorption. Concretely, 250 μ L of LPs-DiD (5 mg/ml) were incubated with 250 μ L of various peptides (NFL, BIOT-NFL or FAM-NFL-peptide) at increasing concentrations (5, 10, 20, 40, 60, 80, 100 μ mol/L). At the end of the adsorption the final concentration of LPs was 2,5 mg/mL. Experiments were conducted at least in triplicate.

2.4. Dynamic Light Scattering (DLS) characterization

Physicochemical parameters of liposomal formulations having DiD were characterized using a Malvern Zetasizer Nano Serie DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). For each analysis, hydrodynamic diameter (nm), zeta potential (mV) and polydispersity index (PDI) were measured. LPs without or with DiD (LPs-DiD) were diluted 1/30 (v/v) in MilliQ purified water and the measurements were achieved at 25 °C in triplicate. Additionally, preparations with NFL-peptide, FAM-NFL-peptide and BIOT-NFL-peptide alone at a concentration of 100 μ mol/L and diluted at 1/5 (v/v) in MilliQ purified water were measured at 25 °C in triplicate. Results were presented in mean size (nm) and size according to intensity %.

2.5. Cryo-electron microscopy (CEM)

Liposomes examination was achieved by a cryo-electron microscope (CEM) in the Microscopy Rennes Imaging Center platform (MRic TEM,

Rennes, France) to maintain the lipid bilayer membrane integrity. Briefly, a vitrification of liposomes alone (POPC/CHOL) concentrated at 3.5 mg/mL, or functionalized with the NFL-peptide, BIOT-NFL-peptide, or FAM-NFL-peptide at 250 μ M was performed using an automatic plunge freezer (EM GP, Leica) under controlled humidity and temperature (Dubochet and McDowell, 1981). Each sample was placed to glow-discharged electron microscope grids followed by blotting and vitrification by quick freezing into liquid ethane. Grids were transferred to a single-axis cryo-holder (model 626, Gatan) and were observed using a 200 kV electron microscope (Tecnai G2 T20 Sphera, FEI) equipped with a 4 k x 4 k CCD camera (XF416, TVIPS). Micrographs were acquired under low electron doses using the camera in binning mode 1 and at a nominal magnification of 25,000 X. Three independent experiments with at least 50 acquisitions per sample were done.

2.6. Size Exclusion Chromatography coupled to Ultra-Performance liquid Chromatography system (SEC/UPLC) to determine peptide adsorbed on the liposome surface

The amount of peptide adsorbed on LPs was evaluated based on the size exclusion principle. We used a PROTEIN KW-803 SHODEX HPLC® SEC Column (500 Å, 5 μ m, 8.0 mm I.D. x 300 mm; Shodex), adapted to the UPLC system (Acquity HClass BIO PDA/FLR, Waters), and several parameters were adjusted. The eluent of NaCl at 0.1 mol/L in water was used as mobile phase following an isocratic gradient with a flow rate at 0.18 mL/min. For each injection, a volume of 10 μ L was chosen. The detection of liposome, NFL-peptide and Biot-NFL peaks were achieved by an UV detector at the wavelength of 220 nm, while the peak of NFL-FAM was determined by using a fluorescence detector at the excitation and emission wavelengths of 484 nm and 501 nm respectively (Acquity FLR chA Ex484, Em 501 nm). Calibration curves were designed distinctively for LPs, NFL peptide, FAM-NFL peptide for concentrations ranging from 0.01 mg/ml to 2 mg/ml for LPs, and 5 μ mol/L to 100 μ mol/L for NFL-peptides. For samples of LPs coated with peptides, the area under each peak was calculated and compared to calibration curves. Thus, the amount of peptides bound to LPs was evaluated by separating free peptide from peptide-LPs.

2.7. Evaluation of cellular toxicity of liposomes functionalized or not with the NFL-peptides by Resazurin and LDH assay

The viability of F98 cancer cells was determined through a colorimetric proliferation assay based on cellular metabolic activity via the Resazurin test. F98 cells were seeded in 96-well plates with 5,000 cells per well and incubated for 24 h at 37 °C. Then F98 cells were treated with various amounts of liposomes without NFL-peptides (LPs-control) at 0.01, 0.05, 0.1, 0.2, 0.5, 1, 1.5 and 2 mg/mL for 48 h at 37 °C and 5 % CO₂. Cells were similarly treated with free peptides (NFL and BIOT-NFL) at 5, 10, 20, 40, 60, 80, 100, 200, 300 and 500 μ mol/L. The toxicity of the FAM-NFL peptide could not be assessed by this assay due to the spectral overlap of the wavelengths of fluorescein and the Resorufin compound. The biological effect of liposomes functionalized with the NFL-peptide and NFL-BIOT-peptide (LPs/NFL-peptide, LPs/BIOT-NFL-peptide) was also analyzed. The concentration of liposomes was set at 0.1 mg/ml corresponding to that used for the internalization tests and the concentration of peptides was identical to the range of free peptides (5, 10, 20, 40, 60, 80, 100, 200, 300 and 500 μ mol/L).

After treatments, 100 μ L of Resazurin at a concentration of 0.025 mg/mL diluted in cell medium was added to each well for 3 h and the fluorescence of Resorufin reactive was measured at 590 nm in a SpectraMax M2 multi-scanning spectrophotometer. The detected fluorescence is proportional to the metabolically active cells. In this experiment, negative control was represented by untreated cells in DMEM. Positive control was obtained by treating F98 cells with colchicine (1 μ g/mL) in DMEM.

The toxicity generated by liposomes was also checked via LDH assay

where the principle is based on the detection of lactate dehydrogenase (LDH) activity released. LDH comes from the cytosol of damaged cells and is measured into the supernatant. Before the treatment, the number of cells per well was optimized. Hence, 12,000 cells (F98) per well were seeded in 96-well plates in DMEM classical medium and incubated for 24 h at 37 °C. Then, cells were treated with a range of LPs concentration (0.01, 0.05, 0.1, 0.2, 0.5, 1, 1.5 and 2 mg/mL) and free NFL-peptides (NFL, BIOT-NFL) at 5, 10, 20, 40, 60, 80, 100, 200, 300 and 500 μ mol/L diluted in Dulbecco's modified Eagle's medium without pyruvate (DMEM; Sigma-Aldrich) supplemented with 10 % of Glutamax, 1 % of fetal bovine serum (Sigma-Aldrich), 1 % of non-essential amino acids (Sigma-Aldrich) and 1 % of antibiotics (100X streptomycin/penicillin; BioWest, Nuaille, France) for 48 h at 37 °C and 5 % CO₂. The initial DMEM medium was replaced by DMEM without pyruvate to avoid interaction with LDH release. After treatment, cells were centrifuged at 250 g for 10 min and 100 μ L of supernatant was transposed to new 96-well plates. Then, 100 μ L of reactive mixture (catalyst (250 μ L) + dye solution (11.25 mL) was added in each well for 30 min at RT in the dark. The measure of absorbance was achieved at 490 nm. In this experiment, negative control was represented by untreated cells in DMEM without pyruvate. The maximum LDH release was obtained through positive control where cells were treated with 2 % of Triton X-100 in DMEM without pyruvate.

2.8. Evaluation of LPS-DiD uptake with NFL-peptides in glioblastoma cells by two complementary methods: Flow cytometry (FACS) and confocal analysis

Rat F98 glioblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing Glutamax and supplemented with 10 % of fetal bovine serum (Sigma-Aldrich), 1 % of non-essential amino acids (Sigma-Aldrich) and 1 % of antibiotics (100X streptomycin/penicillin; BioWest, Nuaille, France). To evaluate the uptake by FACS, 400,000 F98 cells per well were seeded in 6-well plates and incubated for 24 h at 37 °C and 5 % CO₂. Cells were treated with LPs-DiD functionalized or not with the different NFL-peptides (NFL-peptide, BIOT-NFL-peptide, and FAM-NFL-peptide) at increasing concentrations (0.01, 0.05, 0.1, 0.2, 0.5 mg/mL of LPs-DiD and 5, 10, 20, 40, 60, 80 and 100 μ mol/L of peptides) for 1 h. Then, the strategic 0.1 mg/mL concentration of LPs-DiD was chosen to assess LPs-DiD uptake.

Following the incubation, cells were washed with 1X DPBS (Dulbecco's Phosphate Buffered Saline; Gibco, Dardilly, France) and with DMEM free. Then, cells were detached with 1X trypsin (Gibco) for 5 min in 37 °C. After cell harvesting, they were centrifuged at 2000 rpm for 5 min, washed with 1X DPBS and finally diluted in 400 μ L of 1X DPBS and labelled with Propidium Iodide (50 μ g/mL, P4867; Sigma-Aldrich) before analysis on a BD FACSCanto™ flow cytometer (Biosciences, New Jersey, USA). Post-acquisition, the results were analyzed using the FlowJo Software (BD Biosciences, Paris, France). Experiments were conducted at least in triplicate where 20,000 cells were measured for each experiment. Results were presented according to % of DiD positive cells and MFI (Median Fluorescence Intensity). The statistical analysis was only done at the highest concentration of NFL-peptides corresponding to the best effect expected.

In parallel, confocal experiments were done using 25,000 F98 cells per well seeded in 24-well plates before a coating with coverslips for 48 h at 37 °C. Same concentrations as the flow cytometry test were used to treat cells for 6 h. Cells were washed three times with 1X DPBS and fixed with 4 % paraformaldehyde (15714; Delta microscopies, Mauressac, France) for 10 min. Fixation of cells was followed by permeabilization and saturation with a solution of 0.2 % Saponin (Saponin, S4521; Sigma-Aldrich) and 5 % Bovine Serum Albumin (BSA, A7030; Sigma-Aldrich) diluted in 1X DPBS for 15 min. In case of BIOT-NFL treatment, the biotin was revealed via an incubation with an Alexa Fluor™ 488 Streptavidin conjugate (S32354; Thermo Fisher Scientific) diluted in 1X DPBS/5% BSA at 1:500 for 1 h at room temperature. Then, the nucleus

of cells was labeled with 4'6-diaminido-2-phenylindole (DAPI, D9542; Sigma-Aldrich) diluted at 1:300 in 1X DPBS for 10 min. Before observations with a confocal microscope (Leica TCS SP8; Leica Biosystems, Nanterre, France), the coverslips were assembled with the Prolong Gold Antifade reagent (P36930; Thermo Fisher Scientific).

2.9. Exploration of liposomes cellular internalization by transmission electron microscopy

F98 cells were sowed in 12-well plates at 100,000 cells per well and were incubated for 24 h at 37 °C and 5 % CO₂. Then, cells were treated at desired concentrations previously established by resazurin viability test. Briefly, cells were incubated with LPS-DiD, LPs-DiD-BIOT-NFL, LPs-DiD-FAM-NFL at 0.1 mg/ml of LPs concentration and 80 μmol/L of NFL-

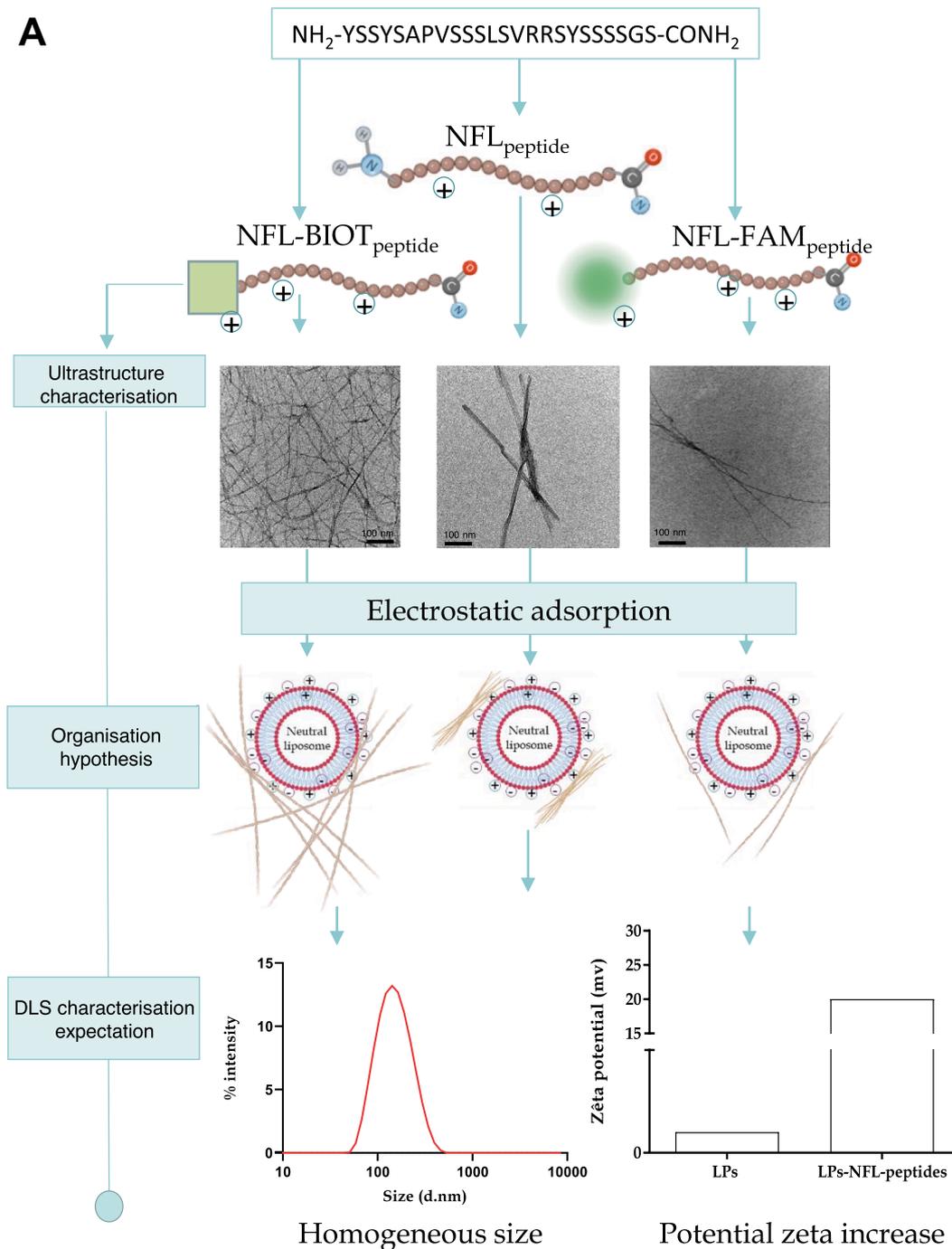


Fig. 1. Schematic representation and characterization of LPs functionalized with NFL-peptides (NFL, FAM, and BIOT). An incubation of 2.5 mg/mL of LPs with 100 μmol/L of peptides was done overnight (around 24 h) at RT under magnetic stirring. Three physicochemical characteristics were measured: the mean size (Z-average) and intensity percent in nanometer (nm), the PDI (polydispersity index) and zeta potential in millivolt (mV). (A) Schematic adsorption process of each peptide coupled to liposomes via electrostatic binding. (B) Zeta potential analysis of LPs alone or LPs decorated with NFL-peptides (LPs-NFL, LPs-FAM-NFL, or LPs-BIOT-NFL). (C) Size measurement in mean size (Z-average) of LPs with or without peptides. Evaluation of LPs diameter according to the intensity percent without adsorption (physical mix / continuous line) or after overnight post adsorption (dotted line). (D) LPs. (E) LPs-NFL. (F) LPs-FAM-NFL. (G) LPs-BIOT-NFL. Errors bars = mean ± SEM. Statistical analysis was executed with Kruskal-Wallis-test (* p < 0.05 and ** p < 0.01, *** p < 0.001). Experiments were achieved at least in triplicate.

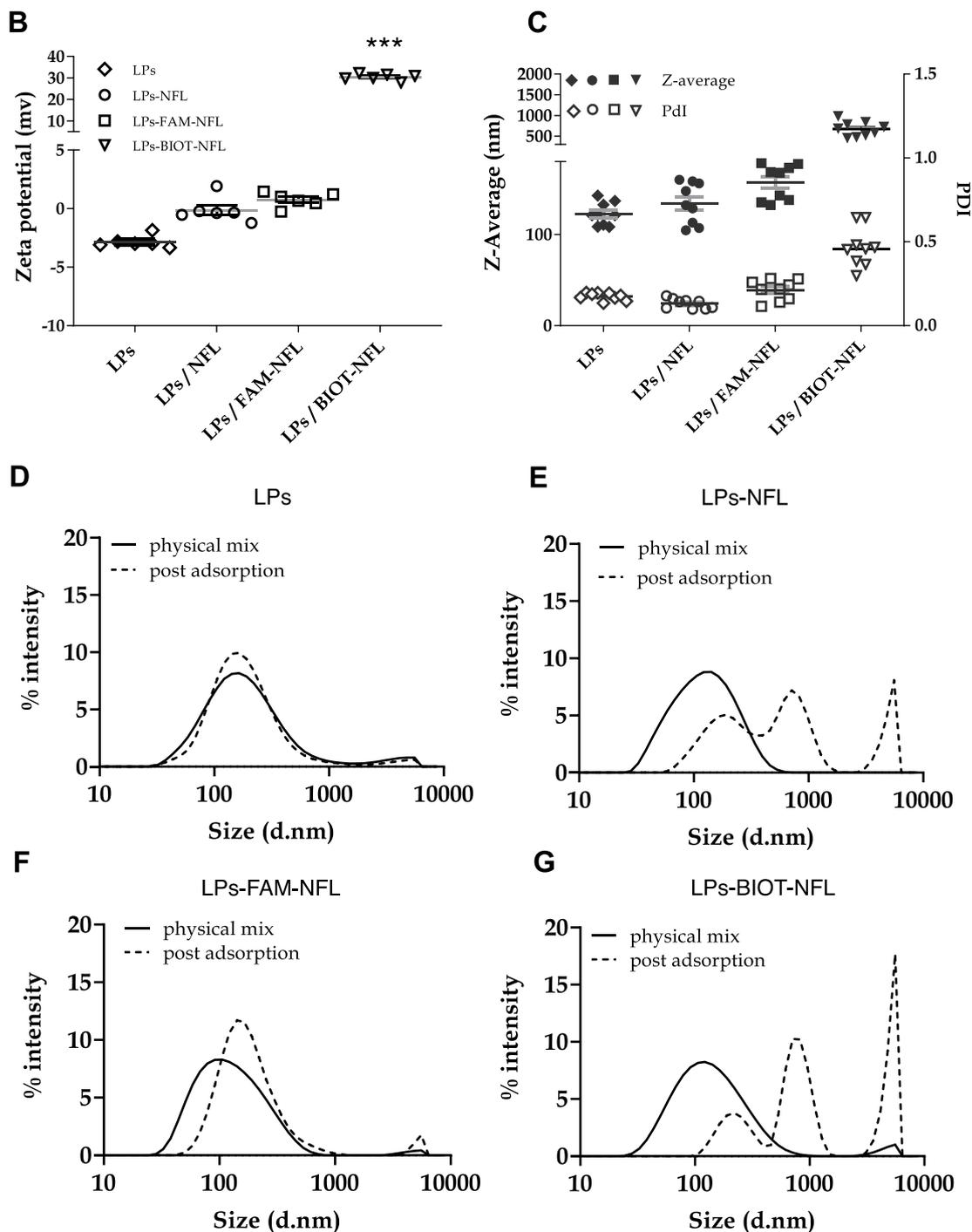


Fig. 1. (continued).

peptides for 4 h at 37 °C and 5 % CO₂. Post incubation, cells were washed with 0.1 M phosphate buffer at pH 7.4 and were fixed with a solution of 2.5 % glutaraldehyde in 0.1 M phosphate buffer, overnight at 4 °C. Then, the fixator was removed, and cells were rinsed with 0.1 M phosphate buffer following by distilled water and post-fixed with a solution of 1 % osmium tetroxide in water for 1 h. Then, cells were rinsed with water (3 times 5 min) and incubated in various ethanol bath: 15 min in 50% ethanol, 15 min in 70% ethanol, 15 min in 95% ethanol and 3 times 30 min in 100% ethanol. After, cells were placed in a solution of 50 % 100% ethanol and 50 % Epon resin mixture (v/v) overnight. The next day, the Epon mixture was removed and replaced by a pure Epon bath for 4 h, replaced by another pure Epon bath 24 h at 37 °C, 24 h at 45 °C

and finally 72 h at 60 °C. After resin polymerization at 60 °C, ultra-fine sections 60 nm thick were made with a UC7 ultramicrotome (Leica, Wetzlar, Germany) and deposited on 150 mesh copper grids. The sections were contrasted with a solution of 3 % uranyl acetate in 50% ethanol for 15 min then rinsed with ultrapure water. Samples were observed using a 120 kV Jeol JEM-1400 electron microscope (Japan) with a SC1000 Orius model832 (Gatan) 4 k CCD camera.

2.10. Monitoring the biodistribution of LPS-DiD in cells via live confocal microscopy

F98 cells were prepared in 8-well Ibidi plates (15 μ-slide 8 well,

80,826 Ibidi GmbH, Munich, Germany), containing 20,000 F98 cells per well, and incubated for 48 h at 37 °C and 5 % CO₂. After incubation, cells were pre-treated with a solution of Green LysoTracker 1 mM diluted 1/1000 in DMEM for 1 h. Then, cells were treated with LPs-DiD at 0,1 mg/mL or LPs-DiD functionalized with NFL-peptide, FAM-NFL-peptide, or BIOT-NFL-peptide at 0,1 mg/mL of LPs-DiD and 25 μmol/L of peptide. Images were acquired in live at different time points after liposomes treatments (15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 24 h) with a confocal microscope in a CO₂ chamber (Leica TCS SP8; Leica Biosystems, Nanterre, France).

2.11. Statistical analysis

All data are represented as mean ± SEM. Experiments were repeated at least three times. Statistical analyzes were performed using GraphPad Prism 7.03 (GraphPad software, San Diego, USA). The asterisks indicate significant level *p < 0.05; **p < 0.01 and ***p < 0.001.

3. Results

3.1. Size, PDI and zeta potential characterization of liposomes decorated by the different NFL-peptides

The adsorption of NFL-peptides including NFL, BIOT-NFL and FAM-NFL on liposomes is mostly due to electrostatic interactions between positive charges of NFL-peptides and the global negative charges exposed on the surface of liposomes (Fig. 1A). As previously documented, the BIOT-NFL-peptide can self-assemble into nanofilaments and the NFL and FAM-NFL-peptide can form short fibrils (Anemeh-Al Ali et al., 2022). Consequently, it is possible to obtain different structural arrangements and organizations of the liposomes with these peptides, which could explain formulations of fairly homogeneous size not or little altered by the adsorption of the peptides and an increase in the zeta potential due to the charges of the peptides (Fig. 1A).

To investigate these possibilities, the interactions between liposomes and NFL-peptide were explored by Dynamic Light Scattering (DLS) through fundamental physicochemical parameters such as size (nm), polydispersity index (PDI) and zeta potential (mV). The size measurement was evaluated according to the mean size (Z-average) and the size correlated to intensity percent. In Fig. 1B, the zeta potential after post adsorption (24 h) was estimated as neutral around $-2 \text{ mV} \pm 0.35 \text{ mV}$, $-0.4 \text{ mV} \pm 1.36 \text{ mV}$ and $0.8 \text{ mV} \pm 0.73 \text{ mV}$ for respectively LPs, LPs-NFL, and LPs-FAM-NFL. Only the zeta potential of LPs-BIOT-NFL was significantly increased with a positive value of $30.8 \text{ mV} \pm 1.86 \text{ mV}$ (Fig. 1B).

To better understand the adsorption mechanism, the zeta potential was measured just after the physical mix (LPs alone, peptides alone, or LPs + peptides), or 24 h later and corresponding to LPs-NFL-peptides adsorbed after 24 h. First, the results showed no significant difference of the NFL-peptides zeta potential measured directly after peptides solubilization or after waiting 24 h (Fig. S1A). The zeta potential was determined as $15.2 \text{ mV} \pm 1.9$, $12.5 \text{ mV} \pm 0.6$, $1.94 \text{ mV} \pm 3.85$, $2.01 \text{ mV} \pm 0.98$, 31.50 ± 2.14 and $32.04 \text{ mV} \pm 2.74$ for respectively NFL (direct), NFL (24 h), FAM-NFL (direct), FAM-NFL (24 h), BIOT-NFL (direct) and BIOT-NFL (24 h). Surprisingly, the size of the BIOT-NFL peptide was systematically higher compared to the two other peptides. Moreover, for all the NFL-peptides, the zeta potential of all physical mix was evaluated as neutral with a measure of $-0.96 \text{ mV} \pm 2.48$, $-0.30 \text{ mV} \pm 0.145$, $-1.55 \text{ mV} \pm 1.97$ for respectively LPs + NFL, LPs + FAM-NFL and LPs + BIOT-NFL (Fig. S1 in Supplementary information).

Concerning the size analysis, the measure of post adsorption formulations after 24 h of incubation revealed a mean size of $152 \text{ nm} \pm 5.78 \text{ nm}$, $161 \pm 12.89 \text{ nm}$, $340 \text{ nm} \pm 3.26 \text{ nm}$ and $678 \text{ nm} \pm 1.96 \text{ nm}$ for respectively LPs, LPs-NFL, LPs-FAM-NFL, and LPs-BIOT-NFL (Fig. 1C). Because of the large size detected, these results suggest that these sizes correspond to long filaments of BIOT-NFL. Therefore, we evaluated the

size distribution via intensity percent without adsorption (physical mix vs LPs + peptides) and measured directly following the mixing of the samples, or 24 h after mixing the samples corresponding to post adsorption. Compared to the mean size analysis, we detected a bigger size showed by a main large peak. As expected, size measurement was almost unchanged for liposomes alone, measured directly after mixing or 24 h later (Fig. 1D). This indicates that the size of liposomes does not change during time. In the opposite, while a unimodal distribution was observed just after mixing the liposomes with NFL, we observed three different peaks 24 h after post adsorption. For LPs-NFL one peak was detected around 150 nm and two at bigger size, approximately 1000 nm, and 6000 nm (Fig. 1E). For LPs-FAM-NFL a major peak was observed around 340 nm and a small intensity peak around 6000 nm (Fig. 1E). Finally, for LPs-BIOT-NFL, a unimodal distribution was observed just after mixing the liposomes with the BIOT-NFL-peptide, but after 24 h there is a small peak around 500 nm and two peaks of greater intensity at 1000 nm and 6000 nm (Fig. 1F).

We noticed also that only one peak was detected with all physical mix (LPS + peptides) measured following the mixture with a size distribution similar to control condition (LPs), which may correspond mainly to liposomes without NFL peptide adsorbed on them. Curiously, DLS analysis was able to detect NFL-peptides size with no major difference between them and with a diameter determined around 300 nm (Fig. S2 in Supplementary information). Interestingly, the comparison between the size measured directly after solubilization of NFL-peptides or 24 h later showed a smaller unimodal distribution for BIOT-NFL-peptide at 24 h (Fig. S2A-C). The sizes were comparable for the NFL-peptide and the FAM-NFL-peptide as well as for the measurements carried out directly after their solubilization or 24 h after. These results can be explained by a disassembly of the BIOT-NFL filaments during magnetic stirring. Taken together, these results suggested that the size and zeta potential of post adsorption formulations (24 h later) reflect mostly the peptides adsorption part on liposomes on their assembly into long filaments at the surface for LPs-NFL and LPs-BIOT-NFL, and not the presence of mostly free peptides around the liposomes for LPs-FAM-NFL.

The liposomes characterization was carried out over a period of six months during which their stability was evaluated. More precisely, physical parameters were measured at different times points (1 month, 3 months and 6 months) following the production of liposomes. Until the 6th month, all formulations were stable without major changes in size, PDI and zeta potential. Interestingly, stability of liposomes was granted for formulations including peptides (Table S1 in supplementary information). The non-modification of size, PDI and zeta potential demonstrated a permanent adsorption of peptides on liposomes.

3.2. Liposomes-peptides analysis using Cryo-Electron microscopy (CEM)

To further investigate the ultrastructural organization of these nanostructures, a cryo-electron microscopy analysis of liposomes-peptides was performed. This technique uses a flash freezing of samples (vitrification) and does not necessitate the use of staining; thus, the samples are very well protected.

Micrographs presented in Fig. 2 showed typical liposomes with a size distribution corresponding to the size intensity percent measured in DLS for each condition corresponding to control LPs (Fig. 2A), LPs-NFL (Fig. 2B), LPs-FAM-NFL (Fig. 2C) or LPs-BIOT-NFL (Fig. 2D). A summary of liposome sizes, ranging from 75 nm to 692 nm and with a mean close to DLS size intensity percent curves, is shown in Table 1. Interestingly, further characterization by CEM revealed no major increase in liposome size despite the functionalization with NFL peptide or BIOT-NFL peptide compared to the control LPs (Fig. 2A-D). Moreover, typical fibrils corresponding to assembled NFL-peptide can be observed along liposome surfaces or together with liposomes (Fig. 2B, 2C and 2D) for a concentration ratio of 3.5 mg/ml of liposome for 250 μmol/L of peptides. Furthermore, dilutions of LPs and LPs-BIOT-NFL were performed in order to better distinguish the localisation of peptides near

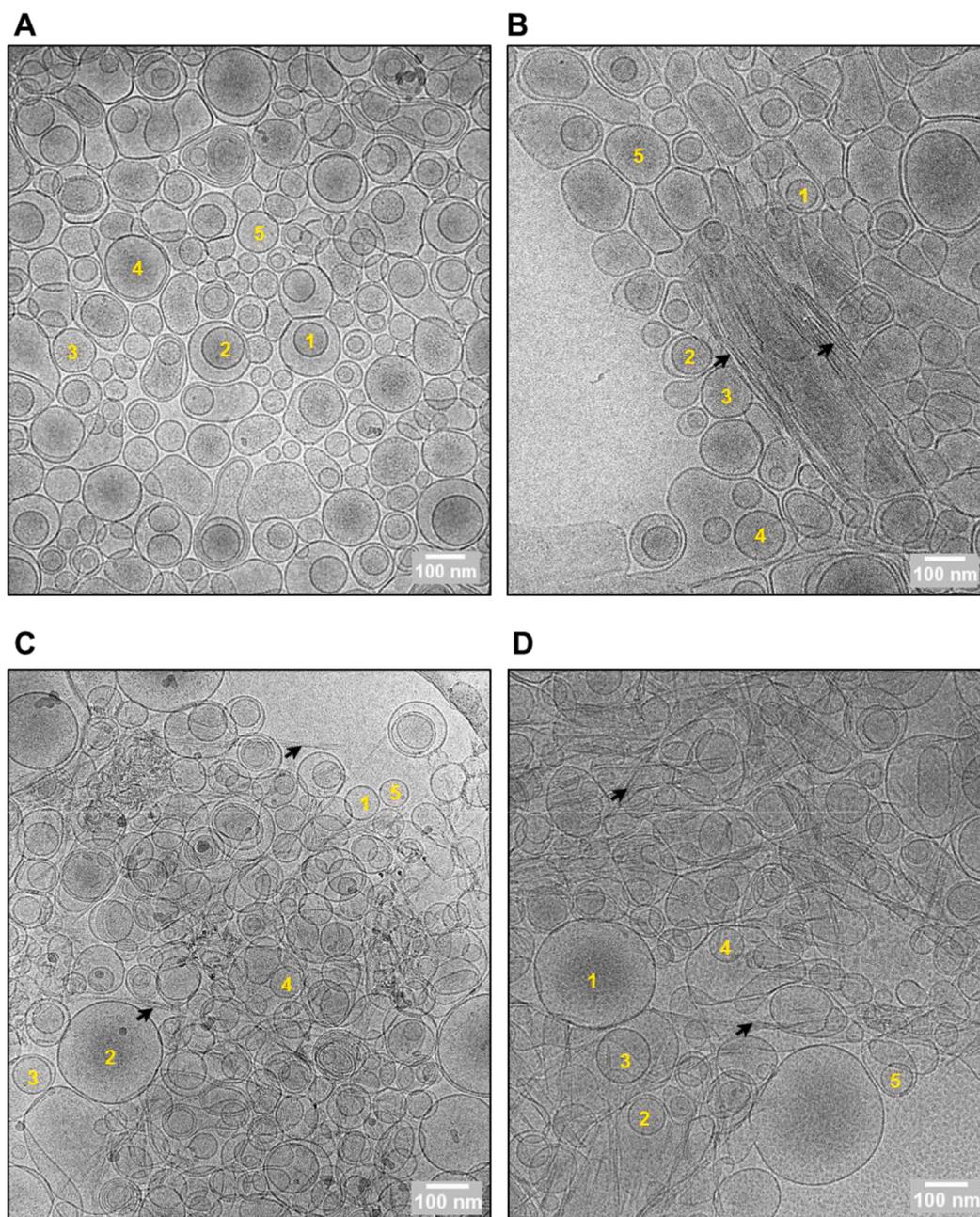


Fig. 2. Micrographs observations by cryo-electron microscopy (CEM) of LPs coupled to NFL-peptides (NFL, FAM, and BIOT). An incubation of 3.5 mg/mL of LPs with 250 $\mu\text{mol/L}$ of peptides was done overnight at RT under magnetic stirring. **(A)** LPs control. **(B)** LPs-NFL. **(C)** LPs-FAM-NFL. **(D)** LPs-BIOT-NFL. Observations were carried out with a cryo-electron microscope. Micrographs were acquired under low electron doses using the camera in binning mode 1 and at a nominal magnification of 25,000X. Scale bar: 100 nm. Observations were achieved at least in triplicate.

liposomes (Fig. S3 in supplemental information). LPs were diluted at 0.7 mg/ml and BIOT-NFL peptide at 50 $\mu\text{mol/L}$. The Fig. S3A shows a better representation of LPs alone which allow to better appreciate their size around 100 nm. Regarding the Fig. S3B, the dilution did not permit to have a better overview of LPs/BIOT-NFL organization. Despite the lowest concentration of the peptide, this peptide induced the aggregation and fusion of plain LPs showing by large LPs estimated around 250 nm. Together, these observations confirmed DLS results and proved the presence of filaments formed by NFL-peptides together with the liposomes.

3.3. Best adsorption interaction between BIOT-NFL peptide and LPs revealed by the size Exclusion Chromatography/Ultra-Performance liquid Chromatography (SEC/UPLC) approach

The interaction of LPs and NFL-peptides was explored by cryo-electron microscopy highlighting the ease with which liposomes can be organized along the peptide filaments. However, these observations did not provide quantitative information. To reinforce the idea of an adsorption existing between LPs and NFL peptides, we evaluated the quantity of peptides on LPs surface after 24 h of stirring by using a Size Exclusion Chromatography (SEC) methodology. Before injecting the samples containing LPs with or without the NFL-peptides through the column, we prepared a range of concentrations for LPs (from 0.01 mg/

Table 1

Size measures of LPs alone or LPs functionalized with the NFL (LPs-NFL), the FAM-NFL (LPs-FAM-NFL) or the BIOT-NFL (LPs-BIOT-NFL). An incubation of 3.5 mg/mL of LPs with 250 $\mu\text{mol/L}$ of peptides was done overnight at RT under gentle magnetic stirring. The mean size (nm) was determined by choosing 5 liposomes from the micrographs. For each CEM picture, the vertical and horizontal diameter of liposome were measured. Experiments were conducted at least in triplicate.

| Conditions | Measure number | Vertical diameter (nm) | Horizontal diameter (nm) | Mean size (nm) |
|--------------|----------------|------------------------|--------------------------|----------------|
| LPs | 1 | 75 | 75 | 100 |
| | 2 | 94 | 94 | |
| | 3 | 96 | 85 | |
| | 4 | 152 | 138 | |
| | 5 | 98 | 96 | |
| LPs-NFL | 1 | 102 | 86 | 142 |
| | 2 | 110 | 119 | |
| | 3 | 155 | 168 | |
| | 4 | 158 | 149 | |
| | 5 | 192 | 179 | |
| LPs-FAM-NFL | 1 | 191 | 194 | 278 |
| | 2 | 640 | 625 | |
| | 3 | 220 | 219 | |
| | 4 | 169 | 184 | |
| | 5 | 170 | 167 | |
| LPs-BIOT-NFL | 1 | 685 | 693 | 312 |
| | 2 | 189 | 206 | |
| | 3 | 303 | 320 | |
| | 4 | 179 | 180 | |
| | 5 | 183 | 177 | |

ml to 2 mg/ml) and NFL-peptides (NFL, FAM-NFL, and BIOT-NFL) (from 5 $\mu\text{mol/L}$ to 80 $\mu\text{mol/L}$) to acquire calibration curves for each component separately. For LPs coated with NFL-peptides, we decided to work at the concentrations tested during uptake experiments. Hence, the concentration of LPs was fixed at 0.1 mg/ml with increasing concentrations of peptides (from 5 $\mu\text{mol/L}$ to 80 $\mu\text{mol/L}$). These analyses are presented in Fig. S4 (Fig. S4 in supplemental information). Among the three peptides, BIOT-NFL shows the strongest interaction with LPs. This was observed from the smallest concentration at 5 $\mu\text{mol/L}$ where the adsorption of 83 % was determined. It is also interesting to note a decrease of the BIOT-NFL amount on LPs surface when increasing concentrations of peptides. In fact, for a ratio of 0.1 mg/ml of LPs and 40 $\mu\text{mol/L}$ of BIOT-NFL, the percent of interaction was only 40 %. Surprisingly, the BIOT-NFL was found to be free in the solution for the highest concentrations (60 and 80 $\mu\text{mol/L}$). This could be explained by the LPs concentration availability which must have been insufficient for having a full peptide adsorption. Regarding the interaction of NFL-peptide and FAM-NFL peptide with LPs, the adsorption profile obtained for the lowest concentrations were comparable. First, it appears that these two peptides were less adsorbed on LPs surface than BIOT-NFL peptide. The highest interaction was estimated at 36 % for 10 $\mu\text{mol/L}$ of NFL-peptide and 49 % for 20 $\mu\text{mol/L}$ of FAM-NFL peptide respectively.

3.4. Acquisition of metabolic and cell lysis profile by Resazurin and lactate dehydrogenase (LDH) assay

The possible toxicity induced by LPs without NFL-peptides (LPs-control) has been evaluated by using Resazurin and LDH assay. These two tests allowed to obtain complementary information. In Fig. 3A, the LDH activity released from the cytosol of cells is expressed as a function of increasing concentrations of LPs-control. This evaluation showed the impact of treating cells with high concentrations of liposomes. From a concentration of 0.5 mg/mL of liposomes, a switch of cell lysis profile is observed. The toxicity of NFL and NFL peptides was also evaluated and showed similar results as resazurin assay, namely higher toxicity of BIOT-NFL peptide than NFL peptide (data not shown). At higher concentrations of control LP, LDH is actively released by cells, leading to cell damage and rendering cells nonviable. These results are correlated with

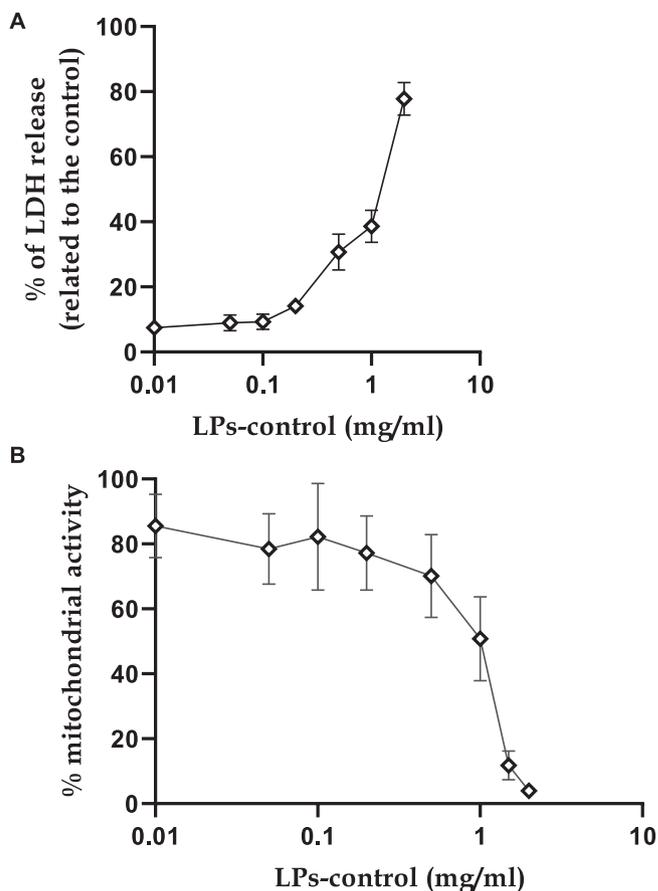


Fig. 3. Evaluation of LPs-control cytotoxicity on F98 cells by Resazurin and LDH assay. The analysis of cytotoxicity induced by LPs without NFL-peptides (LPs-control) on F98 cells was determined by using two different methods. F98 cells were treated with LPs-control at 0.01, 0.05, 0.1, 0.2, 0.5, 1 and 2 mg/ml for 48 h at 37 °C. (A) The cell lysis profile of F98 cells was obtained by using a lactate dehydrogenase Kit (LDH Kit). Results are presented related to the control (untreated cells). (B) The Metabolic activity of cells was evaluated via Resazurin assay. The IC₅₀ of LPs-control was assessed at 0.57 mg/ml. Experiments were conducted at least in triplicate. Data are represented as mean \pm SEM.

the measurement of mitochondrial activity of F98 cells, which shows a decrease in viable cells at high concentration of LPs-control. The 50 % inhibitory concentration (IC₅₀) was also determined at 0.57 mg/mL (Fig. 3B), and therefore we retained a fixed concentration of liposomes at 0.1 mg/ml. The toxicity study of NFL and NFL-BIOT peptides alone showed a greater decrease in cell viability with BIOT-NFL peptide treatment, revealing an IC₅₀ of 366.2 $\mu\text{mol/L}$ for NFL peptide and 48.10 $\mu\text{mol/L}$ for the BIOT-NFL peptide. (Fig. S5 in Supplementary Information). Figure S5 indicates a similar viability profile of F98 cells when they were treated with the peptide-functionalized liposomes (LPs/NFL-peptide, LPs/BIOT-NFL peptide). The IC₅₀ was determined respectively at 383.4 and 47.06 $\mu\text{mol/L}$ (Fig. S5 in supplemental information). These results indicate that toxicity in F98 cells is only induced by peptides and not liposomes.

3.5. Analysis by flow cytometry of the cellular uptake of liposomes alone or functionalized with NFL-peptides

To investigate the possible contribution of NFL peptides to the cellular internalization of liposomes, rat glioblastoma F98 cells were treated with control LPs-DiD or LPs-DiD functionalized with increasing concentrations (5, 10, 20, 40, 60, 80 and 100 $\mu\text{mol/L}$) of peptides (NFL, BIOT-NFL, and FAM-NFL) for a fixed concentration of liposome-DiD

(0.1 mg/mL). The 0.1 mg/mL liposome concentration was selected because no major toxicity was observed following a Resazurin toxicity test and LDH on these F98 cells (Fig. 3).

Quantitative analysis by flow cytometry showed better internalization of LPs-DiD when functionalized with higher concentrations of peptides (NFL, FAM-NFL, and BIOT-NFL) (Fig. 4). In fact, the median fluorescence intensity (MFI) in F98 cells increased proportionally to peptide concentration (from 5 μmol/L to 100 μmol/L). The best intracellular entry of liposomes was observed at the highest concentration of

FAM-NFL peptide (100 μmol/L) (73772 ± 8343 vs 6583 ± 438 for respectively LPs-DiD-FAM-NFL vs LPs-DiD) (Fig. 4B). These experiments demonstrated the important role of the NFL peptides (NFL, FAM-NFL, and BIOT-NFL) to enhance the LPs-DiD internalization into rat glioblastoma cells. Together, these results demonstrate that the presence of NFL peptide allow increased cellular internalization of liposomes.

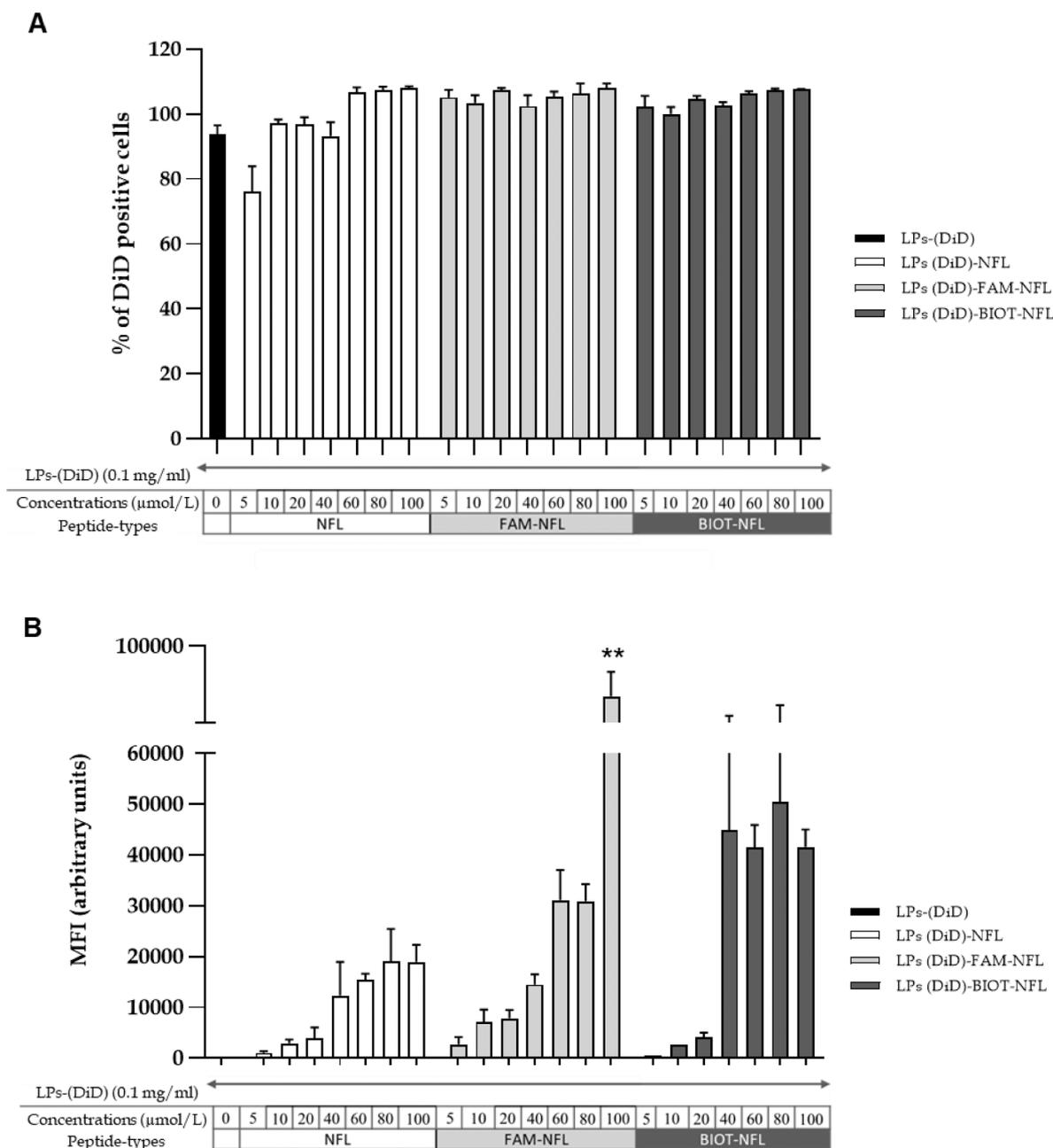


Fig. 4. Cellular uptake evaluation of LPs-DiD functionalized with NFL-peptides including NFL-peptide, FAM-NFL-peptide, and BIOT-NFL-peptide. The quantification of DiD positive cells and MFI (Median Fluorescence Intensity) was determined by flow cytometry on F98 cells treated with LPs-DiD at (0.1 mg/ml) without or with NFL-peptides at (5, 10, 20, 40, 60, 80 and 100 μmol/L) for 1 h at 37 °C. The percentage DiD intensity represent the number of DiD positive cells whereas the MFI signify the DiD intensity captured per cell. (A) F98 cells were incubated with LPs-DiD, LPs-DiD-NFL, LPs-DiD-FAM-NFL, or LPs-DiD-BIOT-NFL; the % DiD positive cells was measured by flow cytometry. (B) MFI quantification by flow cytometry. Experiments were conducted at least in triplicate, and 20,000 cells were measured for each experiment. Data are represented as mean ± SEM. Statistical analysis was performed with a Kruskal-Wallis test following by a complementary test (*p < 0.05, **p < 0.01 and ***p < 0.001). A significant difference (****) was observed between the conditions, emphasized for the LPs-DiD-FAM-NFL at 100 μmol/L of peptide regarding to the control (**).

3.6. Confocal microscopy and TEM confirmation of improved LPS-DiD entry into GBM cells enhanced by NFL-peptides

To validate and further investigate the flow cytometry results, confocal microscope analysis was conducted using the same concentration of LPS-DiD (0.1 mg/mL) alone or adsorbed with NFL-peptides at 40 $\mu\text{mol/L}$. An additional concentration (80 $\mu\text{mol/L}$) was chosen for the LPS-DiD-FAM-NFL formulation to detect the fluorescence of the FAM-NFL-peptide. At higher concentrations the DiD fluorescence signal was saturated and did not allow to obtain quality pictures.

In general, we observed that LPS-DiD can enter in F98 cells with or without peptides functionalization. However, the DiD fluorescence

intensity was systematically higher when cells were incubated with LPS-DiD functionalized with NFL-peptides (NFL, FAM, or BIOT). Similar to the flow cytometry quantification, the best LPS-DiD cellular internalization was observed with the LPS-DiD-FAM-NFL and LPS-DiD-BIOT-NFL (Fig. 5C-E). In addition, it is possible to detect the fluorescence of BIOT-NFL-peptide in green at 40 $\mu\text{mol/L}$ of peptide and its overlay with LPS-DiD produced a typical yellow colour as observed in Fig. 5E. The fluorescence of the FAM-NFL-peptide was detectable only at 80 $\mu\text{mol/L}$, and colocalization with LPS-DiD was also observed in yellow (Fig. 5D). The confirmation of the of LPS-DiD inside F98 cells was determined by the typical orthogonal projections (on the right and below each confocal picture).

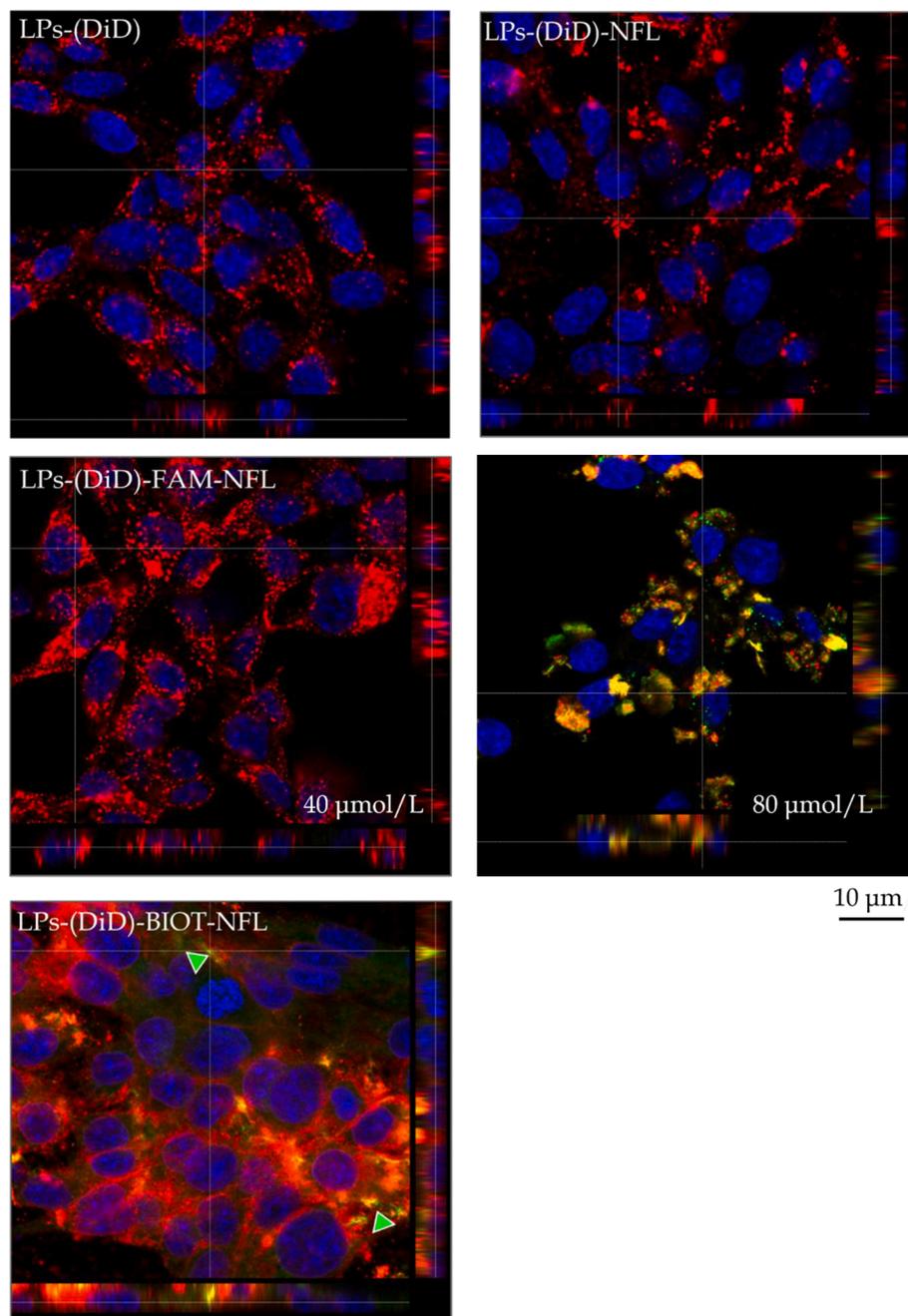


Fig. 5. Confocal investigation to confirm the uptake of LPS-DiD functionalized or not with the NFL-peptides (NFL, FAM, and BIOT) in F98 GBM cells. Cells were incubated 6 h at 37 °C with LPS-DiD at 0.1 mg/mL without or with NFL-peptides at 40 $\mu\text{mol/L}$ of peptide. (A) Control LPS-DiD. (B) LPS-DiD-NFL. (C) LPS-FAM-NFL (40 $\mu\text{mol/L}$). (D) LPS-FAM-NFL (80 $\mu\text{mol/L}$). (E) LPS-BIOT-NFL. Pictures were acquired with a confocal microscope, LPS-(DiD) were labeled in red, the FAM and BIOT-peptide in green, and nucleus in blue. The green arrow indicates the overlay of LPS-DiD and BIOT-NFL-peptide. Experiments were conducted at least triplicate. Scale bar: 10 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

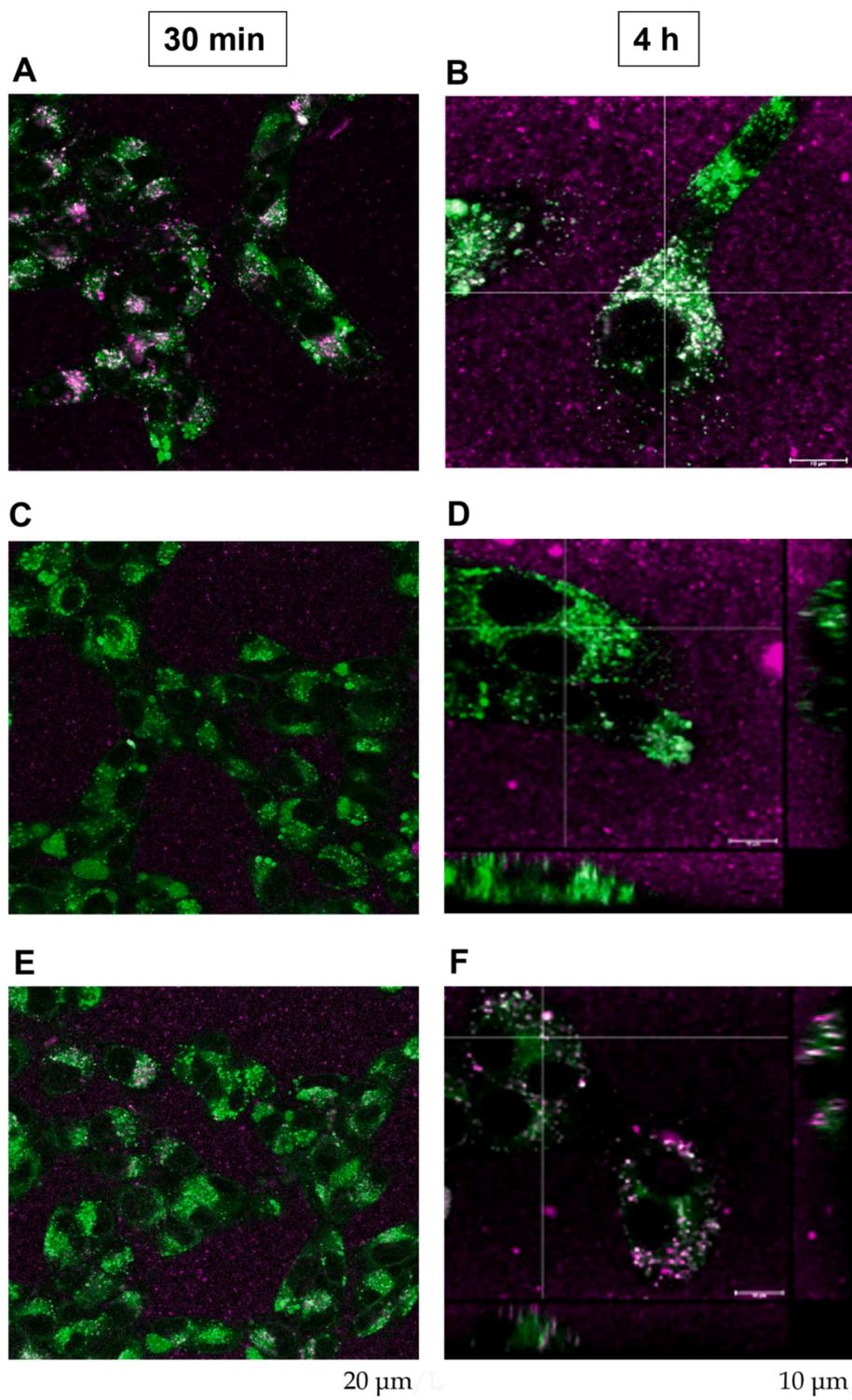


Fig. 6. Biodistribution monitoring of LPS-DiD functionalized or not with the NFL-peptides (FAM, and BIOT) in F98 cells treated with LPS-DiD at 0.1 mg/mL without or with NFL-peptides at 25 µmol/L of peptide. Cells were pre-treated 1 h before the LPS-DiD treatment with a green LysoTracker at 50 µmol/L to label lysosomes. (A) Control LPS-DiD-30 min. (B) Control LPS-DiD-4 h. (C) LPS-BIOT-NFL-30 min. (D) LPS-BIOT-NFL-4 h. (E) LPS-FAM-NFL-30 min. (F) LPS-FAM-NFL-4 h. Images were acquired in live at different times but only two were presented (30 min and 4 h) with a confocal microscope. LPS-DiD were imagined in pink and lysosomes in green. Experiments were conducted at least triplicate. Scale bar: 20 µm and 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells was documented using slices of cells analysed by TEM (Fig. S6 in supplementary information). In this figure, results have been shown for LPS and LPS functionalized with the FAM-NFL peptide. The electron microscope analysis shows that LPS are accumulated in several vacuoles (Fig. S6B) and interestingly they are aligned along FAM-NFL filaments

(Fig. S6C). It is also interesting to note that these vacuoles were observed only following the treatment of cells with LPS or LPS-FAM-NFL, and they were not present in untreated cells.

3.7. LPs-DiD are collocated with lysosomes after 4 h uptake

According to the internalization results obtained above, we explored the bio distribution of liposomes filled with DiD in F98 cells. The cellular lysosomes were labelled with the green LysoTracker 1 h, then a treatment was realized with LPs-DiD functionalized or not with BIOT-NFL-peptide or FAM-NFL peptide. In Fig. 6, two lifetimes have been chosen in order to emphasize the different levels of lysosomes uptake. At 30 min, the LPs-DiD were already internalized by F98 cells (Fig. 6A) and this uptake continued to increase up to 4 h, where some LPs-DiD were collocated with lysosome organelle (Fig. 6B). This collocation is visible and is corresponding to the white spot overlay. If we focused on the LPs-DiD-BIOT-NFL biodistribution, we observed a difficulty to penetrate into GBM cells with poor LPs-DiD present inside the cells (Fig. 6C-D).

When liposomes were functionalized with the FAM-NFL-peptide a strong presence of LPs-DiD in lysosomes of F98 cells was observed at 4 h (Fig. 6F). Compared to the control LPs-DiD, no or poor uptake was observed at 30 min with the NFL-peptide conditions adsorbed on liposomes (Fig. 6C-E). Regarding the confocal analysis microscopy experiment previously described, we could explain these results by the fact that we worked with a live and not a stained GBM cells model deprived in CO₂ during the confocal acquisitions. These experiments were conducted at least in triplicate. Scale bars = 20 and 10 μm.

4. Discussion

Recently, the investigation of the NFL-peptide ultrastructure provided a better understanding of its physical structure based on self-assembly process, for the BIOT-NFL-peptide (Alnemeh-Ali et al., 2022). It also allowed to describe its structural organisation when it was associated with nanoparticles such as lipid nanocapsules (Griveau et al., 2022). In this study, we have focused on liposomes organisation without or with NFL-peptides adsorption on their surface. To achieve this study, we used two main complementary methods including DLS and cryo-electron microscopy. The measure of size determined by DLS showed a difference depending on the type of unit chosen, with the majority of the larger size expressed in percentage of intensity compared to mean size (nm) (Fig. 1). Moreover, the comparison between a physical mix composed of liposome on one side, and peptides on another side, allowed to prove the interaction between NFL-peptide and liposome, specifically for BIOT-NFL-peptide (Fig. 1G).

In fact, the size and zeta potential determined after adsorption process validated mostly the presence of NFL-peptide at the liposome surface and poorly the free peptides. Moreover, the bigger size obtained with liposome-BIOT-NFL highlighted an important formation of filaments and the interaction of the peptide with liposomes. A study proved the necessity to control the concentration ratio between lipids in liposome bilayer membrane and peptide adsorption (Nicol et al., 1996). This investigation showed the involvement of high cholesterol concentration for a strong peptide aggregation. In our study, we only fixed a unique ratio between POPC and CHOL (70/30). For further investigation, it could be interesting to vary this ratio in order to determine if increased NFL-peptide adsorption would occur. Additionally, Nicol et al. correlated the type of peptide configuration with the nanoparticle interaction. Results concerning the HIV-1 fusion peptide showed a preference of this peptide to adopt β sheet configuration at high concentration inducing a better adsorption unlike the alpha helix configuration (Nicol et al., 1996). The circular dichroism investigation of NFL-peptide indicated that the peptide is adopting two main configurations: β-sheets and alpha-helical structures (Berges et al., 2012). Interestingly, when the BIOT-NFL-peptide is self-assembled, it is adopting mainly the β sheet structure as indicated by X-ray scattering (Alnemeh-Ali et al., 2022).

The DLS method was also able to detect an own size and zeta potential of NFL-peptide, FAM-NFL-peptide, and BIOT-NFL-peptide (Fig. S2). However, we were surprised not to detect two peaks right after mixing the liposomes with the peptides. This could indicate a limit

of DLS measurements when larger molecules can hide smaller ones.

The liposomes stability is an essential criterion in the process of developing liposomes such as drug delivery system for pharmaceutical study. This stability could be explored under several parameters including the chemical and physical stability, the activity of encapsulated drugs and their interaction with biological fluids (Monteiro et al., 2014). Among physical parameters, the evaluation of size is a standard way to determine liposomes stability. In our study, the control of liposomes diameter covering a period of six months has confirmed the stability of formulations. Over this time, we observed no major size modifications revealing no aggregations of liposomes with or without NFL-peptides. These stability data are important and promising for the next steps in encapsulating drugs.

The use of cryo electron microscopy was motivated by the advantage of this method to protect samples, in particular liposomes from drying alteration. In the Fig. 2 micrographs demonstrated that the particle diameter was closer to the measures obtained according to intensity percentage in DLS and allowed to confirm the heterogeneous liposome formulations. However, it was difficult to determine the disposition of liposomes regarding the peptide filaments, in particular for LPs-BIOT-NFL (Fig. 2D) due to the presence of too many nanofilaments. Based on this information, samples were diluted to prevent the formation of long filaments, or aggregation of filaments. As shown in Fig. S3B, the BIOT-NFL peptide self-assembled inducing the formation of nanofilaments despite a smaller concentration. This phenomenon is mainly observed for the BIOT-NFL peptide which led to liposomes aggregation and fusion (Fig. S3B). The fusion of liposome in contact of peptides was already described through the example of HIV-1 fusion peptide (Nir and Nieva, 2000). This work demonstrated that the fusion of liposomes was promoted by a larger size and higher concentration of peptides. Additionally, the conformation of peptide played a significant part of LPs fusion where the α conformation promoted the leakage of LPs membrane while the β conformation is involved in the vesicle fusion.

This work highlighted the importance of using different methods for a better characterization when elaborating functional nano-systems. A recent review challenged this topic by showing the numerous limits of DLS notably imputable to the signal DLS monopolization of big molecules at the expense of small molecules (Hallan et al., 2021).

Every day, in the field of cancer treatment, many proposals emerge with the common goal of becoming the best possible answer. Among the range of suggestions, a vehicle like liposome associated to peptides is regularly employed in the spectrum of cancer therapy. Recently, Sonju et al. have gathered a large number of articles demonstrating the beneficial role of this type of nanosystem for targeting tumour cells (Sonju et al., 2021). In the internalization process, these studies revealed the importance of having peptides at LPs surface, which is potentiating the entry of liposomes in tumor cells. This mechanism is often demonstrated *in vitro* by focusing the efforts on liposomes detection and quantification. However, few studies have dedicated their research on the physicochemical interaction between peptides and liposome. In this study, we brought another perspective by establishing a link between the ultrastructural conformation of LPs decorated with peptides and their *in vitro* uptake. Our results clearly indicate that adjusting the LPs/peptides ratio represents an essential lever to obtain the optimal cellular internalization.

Before the internalization study, the toxicity of plain LPs was determined by Resazurin and LDH assay (Fig. 3). These two experiments carried out in parallel allowed to show a limit of using plain LPs on F98 cells due to their important toxicity at high concentrations. The IC₅₀ was assessed at 0.57 mg/ml. In the literature, several data are correlated with those we obtained. For example, a team tested a formulation of DPPC-based cationic liposomes on F98 cells and determined the median lethal concentration (LC₅₀) at 6.07 μmol/L (Shi et al., 2018). The phospholipid DPPC is chemically close to the POPC that we used in our study and the concentration of 0.57 mg/ml corresponds to 8.91 μmol/L. These toxicity experiments confirm the data found in literature.

Interestingly, the BIOT-NFL-peptide exhibited a higher toxicity to GBM cells than NFL-peptide (Fig. S5). These results highlight the importance of continuing to explore the relationship between the mechanistic aspects of the BIOT-NFL-peptide (self-assembly into nanofilaments) and its biological effects on tumor cells.

The exploration of the role of NFL-peptides in the cellular uptake of liposomes was built on previous experiments conducted in the laboratory. The NFL peptide was already coupled to various types of nanoparticles such as lipid nanocapsules, gold nanoparticles, or magnetic porous silicon nanorods and showed the capacity of this peptide to increase the cellular entry of nanovectors in several cell lines models (Balzeau et al., 2013; Karim et al., 2018; Chaix et al., 2022; Griveau et al., 2022; Arib et al., 2022). In the present work, we have intentionally set the LPs-DiD concentration at 0.1 mg/mL to avoid any toxicity and to evaluate the cellular uptake potential of NFL-peptides. If we consider, the percentage of LPs-DiD uptake regarding non-individual cells, the flow cytometry results showed no major differences between LPs-DiD used as control and LPs-DiD-NFL, LPs-DiD-FAM-NFL, LPs-DiD-BIOT-NFL (Fig. 4A). One explanation could be that every cell is internalizing similarly LPs-DiD. Nevertheless, the examination of LPs-DiD cellular uptake revealed that NFL-Peptides increased the intensity of LPs-DiD for a specific cell as the peptide concentration increase, indicating probably an increased number of fluorescent liposomes in each cell. The highest effect was observed with BIOT-NFL-peptide (Fig. 4B). The confocal microscopy results confirmed the internalization study obtained by flow cytometry (Fig. 4). Herein, we confirm the role of NFL-peptide as an effective targeting molecule for GBM cells.

Other studies have demonstrated the benefit of using peptides to target glioblastoma cells. Mander et al, used a tumour targeting cell penetrating peptide (CPP) called p28 in association with the conventional GBM treatment Temozolomide. Thanks to p28 addition, it was possible to enhance the cytotoxicity effect of the drug tested on L-229 and U-118 cells (Mander et al., 2022). CPP are often used with liposomes as an effective drug delivery system in GBM context. In 2013, Yang et al, investigated an RGERPPR peptide with affinity for the neuropilin-1 receptor. Combined with pegylated and Doxorubicin-filled liposomes, this peptide has been shown to improve the penetration of such liposomes into tumour vessels and the reduction of tumour mass (Yang et al., 2013).

In the GBM therapy, the passage of BBB is one of the main obstacle. We do not have analysed this aspect for our nanosystem, but several inputs are already published. The BBB issue could be bypassed by using dual liposome targeting with on one side a peptide with penetrating power (CPP) and on the other side a specific targeting peptide. In this context, Han et al, developed liposomes with dual ligands involving TAT which is the most common CPP and Angiopep-2 peptide that specifically targets the low-density lipoprotein receptor protein 1 (LRP1), a receptor overexpressed on BBB. Through this combination, the liposomes were better absorbed in human U251 GBM cells inducing cellular damage such as nuclear envelope disruption, mitochondria decrease and necrosis state (Han et al., 2017). This strategy could be considered in the same way with the NFL-peptide as the main CPP, with another peptide targeting specifically the BBB.

For ethical reasons in animal experimentation, our work was limited to cellular models, but it clearly shows the growing interest in using functionalized nanovectors, in particular here liposomes, to better target cancerous or pathological cells and allow massive entry of such nanovectors.

5. Conclusion

This study illustrates the different ultrastructural organisations of NFL-peptides when functionalizing liposomes. The BIOT-NFL-peptide is the most adsorbed at the liposome surface depending on its faculty to produce nanofilaments. All NFL-peptides demonstrate their key properties to increase liposomes uptake into glioblastoma cells. Finally, the

association of NFL peptides to liposomes provide a promising targeting drug delivery system to target GBM cells.

CRedit authorship contribution statement

M-A. Jourdain: Methodology, Formal analysis, Investigation, Resources, Validation, Writing – original draft. **A. Dupont:** Investigation. **N. Lautram:** Investigation. **J. Eyer:** Conception, Investigation, Resources, Writing, Reviewing and Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2024.123805>.

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