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NFL-lipid nanocapsules for brain neural stem cell targeting *in vitro* and *in vivo*

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ABSTRACT

The replacement of injured neurons by the selective stimulation of neural stem cells *in situ* represents a potential therapeutic strategy for the treatment of neurodegenerative diseases. The peptide NFL-TBS.40-63 showed specific interactions towards neural stem cells of the subventricular zone. The aim of our work was to produce a NFL-based drug delivery system able to target neural stem cells through the selective affinity between the peptide and these cells. NFL-TBS.40-63 (NFL) was adsorbed on lipid nanocapsules (LNC) whom targeting efficiency was evaluated on neural stem cells from the subventricular zone (brain) and from the central canal (spinal cord). NFL-LNC were incubated with primary neural stem cells *in vitro* or injected *in vivo* in adult rat brain (right lateral ventricle) or spinal cord (T10). NFL-LNC interactions with neural stem cells were different depending on the origin of the cells. NFL-LNC showed a preferential uptake by neural stem cells from the brain, while they did not interact with neural stem cells from the spinal cord. The results obtained *in vivo* correlate with the results observed *in vitro*, demonstrating that NFL-LNC represent a promising therapeutic strategy to selectively deliver bioactive molecules to brain neural stem cells.

1. Introduction

Stem cells are characterized by self-renewal [1] and differentiation into specialized cells [2]. Stem cell classification can be related to their developmental stage (embryonic, foetal or adult) [3], their plasticity (totipotent, pluripotent, multipotent or unipotent) [4] or their anatomical localization [5]. In the adult mammalian organism, neural stem cells (NSC) are multipotent self-renewing cells, localized in the central nervous system, and able to differentiate into neurons, astrocytes and oligodendrocytes [6]. NSC are confined in places called niches, like the subventricular (SVZ) and the subgranular zone in the brain [7], or the central canal (CC) in the spinal cord [8]. Neurogenesis can occur during the adulthood in these niches [9]. NSC can be isolated and cultivated as floating aggregates named neurospheres [10]. The absence of growing factors (EGF and FGF) as well as the presence of serum induces NSC to differentiate into specialized neuronal cells [11]. The regenerative potential of these cells [12], and their differentiation property, make them a suitable tool for the treatment of neurodegenerative diseases [13]. NSC can potentially replace and repair injured neurons [14]. The most common NSC-based therapeutic approach consists in allogenic NSC transplantation at the site of interest [15–18]. Unfortunately, this method is strongly limited by the dangers of their transplantation (development of metastases, immune-mediated rejections, death of the transplanted cells [19]), and by their availability [20]. Thus, new strategies have emerged aiming at the *in situ* stimulation of endogenous NSC to induce their differentiation [21,22]. While this approach would solve most of the issues associated with NSC transplantation, the lack of a selective NSC targeting strategy greatly slows the progresses in that direction.

It has been recently shown that NFL-TBS.40-63 (NFL), a synthetic 24-aminoacid peptide corresponding to the tubulin-binding site of the neurofilament light subunit [23], targets NSC of the SVZ (SVZ-NSC) [24]. *In vitro*, NFL penetrates massively in SVZ-NSC by direct translocation increasing their adhesion and differentiation, but it enters poorly in astrocytes and neurons. *In vivo*, following injection in the rat brain lateral ventricle, the peptide localizes specifically in the SVZ-NSC niche. NFL induced no major toxicity *in vitro* and *in vivo*. NFL was also tested as anti-

Abbreviations: CC, central canal; CC-NSC, neural stem cells of the central canal; CPP, cell-penetrating peptides; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine 4-chlorobenzenesulfonate; fluoVIM, 5-FAM-labeled VIM-TBS.58-81; LNC, DiD-loaded lipid nanocapsules; fluoNFL, 5-FAM-labeled NFL-TBS.40-63; fluoTAT, 5-FAM-labeled TAT.48-60; NFL, biotinylated NFL-TBS.40-63; NFL-LNC, NFL-functionalized DiD-loaded lipid nanocapsules; NFL-nLNC, NFL DiD-loaded neutral lipid nanocapsules; nLNC, neutral DiD-loaded lipid nanocapsules; NSC, neural stem cells; SCR-NFL, 5-FAM-labeled scramble NFL; SVZ-NSC, neural stem cells of the subventricular zone; SVZ, subventricular zone; TAT, biotinylated TAT.48-60; TAT-LNC, TAT-functionalized DiD-loaded lipid nanocapsules; VIM, biotinylated VIM-TBS.58-81..

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glioblastoma drug, alone [25,26] or associated with nanoparticulate systems [27]. The active uptake of the peptide by cancerous cells induced the inhibition of the tumor growth by disrupting their microtubule and mitochondrial networks [25,28]. Such cytotoxic effect was not observed on healthy cells. The peptide also showed *in vitro* affinity for oligodendrocytes, in which it penetrates by clathrin-dependent endocytosis, promoting their cellular growth and survival [29,30]. The reason of the affinity between NFL and these different cells (SVZ-NSC, glioblastoma cells and oligodendrocytes) is not well understood, neither the reason of the different mechanisms of penetration (passive in the first case and active in the second and third case). Physicochemical investigation on NFL indicated a strong structure/activity correlation (by alanine-scanning assay) and a dependency of the ratio alpha helix/beta sheet to the environmental conditions (by circular dichroism) [31]. Although NFL has high affinity for SVZ-NSC, it did not induce a yet identified therapeutic effect. Moreover, the strong structure/activity correlation [31] limits the chemical coupling of bioactive molecules on NFL that would induce SVZ-NSC differentiation. While a direct therapeutic application of the peptide is still not excluded, its association with a drug delivery system could potentially be a useful strategy for selective delivery of bioactive molecules to NSC.

Lipid nanocapsules (LNC) are the drug delivery system that has been selected for this purpose. The excipients used to produce LNC are approved by the FDA (for oral, topical and parenteral administration). The formulation is highly stable and the process of production is organic solvent-free and follows a phase inversion temperature method [32–35]. LNC are a versatile vector promoting the nano-encapsulation or the nano-association of a wide range of therapeutics (hydrophilic, lipophilic or amphiphilic [36]). Furthermore this nanoparticulate system can be easily traceable by fluorescent labelling. DiD is one of the most used fluorescent dye for LNC tracking [37] and its compatibility for *in vivo* experiment (*e.g.* intra-lateral ventricle injection in the brain) is well known [38]. NFL was recently related to the cell-penetrating peptides (CPP) [26], due to the characteristics it shares with them, including the positive charge, the low molecular weight and the balance between endocytosis and direct translocation in cell penetration [39]. CPP have been successfully used to enhance the cellular delivery of a large variety of cargos including nanoparticles [40]. Hence we decided to compare LNC presenting NFL at their surface (NFL-LNC) with LNC decorated with other described CPP such as TAT [41] and VIM [42].

The objective of this work was to evaluate whether the LNC-based drug delivery system is able to selectively interact with NSC. NFL-LNC targeting efficiency was tested *in vitro* on NSC isolated from the SVZ (brain), or from the CC (spinal cord). The mechanism of interaction between NFL-LNC and NSC was characterized *in vitro*. Then, NFL-LNC were injected in the adult rat brain (right lateral ventricle), or in the spinal cord (T10) to determine if they specifically target endogenous NSC.

2. Materials and methods

2.1. Materials

Biotinylated NFL-TBS.40-63 (NFL), biotinylated VIM-TBS.58-81 (VIM), biotinylated TAT (TAT), 5-FAM-labeled scramble NFL (SCR-NFL), 5-FAM-labeled TAT (fluoTAT), 5-FAM-labeled NFL-TBS.40-63 (fluoNFL) and 5-FAM-labeled VIM-TBS.58-81 (fluoVIM) were purchased from GeneCust (Luxembourg, Luxembourg) (see Supplemental data 8). Labrafac® was purchased from Gattefosse SA (Saint-Priest, France). Lipoïd® was purchased from Lipoid Gmbh (Ludwigshafen, Germany). Solutol HS was purchased from BASF (Ludwigshafen, Germany). Sodium chloride (NaCl) was purchased from Prolabo (Fontenay-sous-bois, France). D-glucose, Phalloidin-Rhodamine and primary antibodies mouse anti-alpha tubulin, anti-nestin, anti-GFAP and anti-vimentin were purchased from Sigma (Saint-Louis, Missouri). DiD' solid; DiIC18(5) solid (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt) (DiD), secondary antibodies anti-mouse Alexa Fluor 488, Alexa Fluor 568 and DAPI, HEPES, Pen/Strept, Na Pyruvate, B27, MEM alpha (no nucleosides), 0.05% Trypsin-EDTA×, DNAse and ProLong Gold antifade were purchased from Thermo Fisher Scientific (Waltham, Massachusetts). EGF and bFGF were purchased from Tebu-Bio (Le Perray en Yvelines, France). BD CellTak was purchased from Corning Inc. (New York, New York). BCA Uptima was purchased from Interchim (Montluçon, France). CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega (Fitchburg, Wisconsin). Amicon Ultra-0.5 ml 100 K filters were purchased from Merck Millipore (Billerica, Massachusetts). The isolation of NSC and *in vivo* experiments were performed according to Directive 2010/63/EU, to guidelines of the French and Belgian Government following the approval by the local committee of Pays de la Loire for Ethic on Animal Experiments or by the ethical committee for animal care of the faculty of medicine of the Université catholique de Louvain.

2.2. Preparation and characterization of LNC presenting CPP at their surface

2.2.1. Preparation of DiD-labeled lipid nanocapsule stock solution (stock-LNC)

Stock-LNC was prepared according to Heurtault et al. [32]. Briefly, 0.846 g Solutol HS15, 0.075 g Lipoïd®, 0.089 g NaCl, 1.028 g Labrafac® and 2.962 ml of water were mixed under magnetic stirring for 5 min at 30 °C. Temperature cycles (a minimum of 3) of progressive heating/cooling were done between 60 °C and 90 °C. During the cooling of the last cycle, at 80 °C 27.5 μl of DiD solution (1 mg/ml in absolute ethanol) and at 74 °C 12.5 ml of cold water were respectively added, under high speed stirring. The nanoparticles were filtered with a 0.2 μm filter and stored at 4 °C (Supplemental data 7).

2.2.2. Preparation of CPP DiD-labeled lipid nanocapsules (CPP-LNC)

TAT-, VIM- and NFL-LNC were produced by incubating 369 μl of 1 mM peptide solution (in water) overnight with 1 ml of stock-LNC under gentle stirring. LNC without peptide (negative control) were produced incubating 369 μl of water overnight with 1 ml of DiD-labeled lipid nanocapsule stock solution under gentle stirring.

2.2.3. Physicochemical properties of CPP-LNC

The size, z-potential and PDI of CPP-LNC were characterized using a Malvern Zetasizer Nano Serie DTS 1060 (Malvern Instruments). For the measurement of size and PDI, nanoparticles were diluted 1/100 (v/v) in water. For the measurement of z-potential, nanoparticles were diluted 1/100 (v/v) in NaCl 10 mM. Peptide concentration at LNC surface was indirectly measured by BCA Uptima quantification [27]. Briefly, CPP-LNC were filtered by centrifugation at 4000*g* during 30 min using an Amicon Ultra-0.5 ml 100 K filter. The water phase containing the unbound peptide was collected, and the amount of unbound peptide was measured. The positive controls consisted of free peptide (NFL, TAT or VIM), and the negative control was LNC alone.

2.2.4. Characterization of the interaction between NFL and LNC

NFL-LNC size was measured after dilution 1/100 (v/v) in water or in NaCl 1 M. Neutral LNC (nLNC) and NFL-nLNC were prepared following the same protocol as described above but without Lipoïd®. Size and PDI were measured in different concentrations of Tris or NaCl (0, 0.005, 0.05, 0.15, 0.25, 0.5 and 1 M).

2.3. Impact of CPP on LNC interactions with NSC primary cultures

2.3.1. Isolation of SVZ-NSC

SVZ-NSC were isolated according to Guo et al. [43]. Briefly, new-born rats (1 to 5 day-old) were sacrificed by decapitation. The brain was removed and put in the dissection buffer (1.25 ml of D-glucose 1 M, 750 μl of HEPES and 500 μl Pen/Strept in 50 ml of HBSS medium). It