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H. Alnemeh-Al Ali, A. Griveau, F. Artzner, A. Dupont, N. Lautram, et al.. Investigation on the self-assembly of the NFL-TBS.40-63 peptide and its interaction with gold nanoparticles as a delivery agent for glioblastoma. International Journal of Pharmaceutics: X, 2022, 4, pp.100128. 10.1016/j.ijpx.2022.100128 . hal-03823993

HAL Id: hal-03823993

<https://hal.science/hal-03823993>

Submitted on 23 Jan 2023

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Investigation on the self-assembly of the NFL-TBS.40-63 peptide and its interaction with gold nanoparticles as a delivery agent for glioblastoma

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ARTICLE INFO

Keywords:

NFL-TBS.40-63 peptide
Self-assembly
BIOT-NFL-peptide-nanofibers
Gold nanoparticles
Glioblastoma
Targeted drug delivery

ABSTRACT

NFL-TBS.40-63 peptide is a recently discovered peptide derived from the light neurofilament chain (NFL). In this study, we demonstrated that the Biotinylated-NFL-peptide (BIOT-NFL) can spontaneously self-assemble into well-organized nanofibers (approximately 5 nm width and several micrometers in length) in several solutions, whereas the typical self-assembly was not systematically observed from other peptides with or without coupling. The critical aggregation concentration that allows the BIOT-NFL-peptide to aggregate and auto associate was determined at 10^{-4} mol/L by surface tension measurements. X-ray scattering of BIOT-NFL-peptide also demonstrated its beta-sheet structure that can facilitate the intermolecular interactions involved in the self-assembly process. The possible disassembly of self-assembled BIOT-NFL-peptide-nanofibers was examined via a dialysis membrane study. We further investigated the interaction between nanofibers formed by BIOT-NFL-peptide and gold nanoparticles. Interestingly, a strong interaction was demonstrated between these nanoparticles and BIOT-NFL-peptide resulted in the formation of BIOT-NFL-peptide-nanofibers grandly decorated by gold nanoparticles. Finally, we investigated the internalization of gold nanoparticles coupled with BIOT-NFL-nanofibers into F98 rat glioblastoma cells, which was increased compared to the non-coupled control gold nanoparticles. All these results indicate that this peptide could be a promising therapeutic agent for targeted delivery.

1. Introduction

The development of peptide therapeutics has recently received a huge amount of attention due to their potential for various applications in biomedicine and nanotechnology (Wang et al., 2016). The high biological activity, specificity and biodegradability of peptides make them especially interesting for therapeutic purposes (Vlieghe et al., 2010). NFL-TBS.40-63 peptide is a cell-penetrating peptide derived from the light neurofilament subunit (NFL) and belonging to the tubulin-binding-compounds that represent an important class of anticancer drugs (Berges et al., 2012a). This peptide consists of 24 amino acids corresponding to

the sequence of the tubulin-binding site (TBS) located on the N-terminal domain of NFL between amino acids 40 and 63, and thus, this peptide can interact with tubulin and inhibit the proliferation of glioma cells by disrupting their microtubule network (Berges et al., 2012b). Interestingly, our previous works have shown a preferential uptake of NFL-peptide by several sources of glioblastoma cell lines like human (T98G and U87-MG), rats (9 L and F98), and mouse (GL261) glioblastoma (GBM) cells, as well as human glioma derived stem cells, where it provokes apoptosis without damaging the healthy cells of the nervous system (Berges et al., 2012a; Balzeau et al., 2013; Lépinoux-Chambaud and Eyer, 2019). The selective and massive penetration of NFL-peptide

Abbreviations: NFL-peptide, neurofilament light subunit derived 24 amino acid tubulin-binding site peptide NFL-TBS.40-63; BIOT-NFL, the NFL-TBS.40-63 peptide coupled to the biotin; FAM-NFL, the NFL-TBS.40-63 peptide coupled to 5-carboxy-fluorescein; nude-NFL, the NFL-TBS.40-63 peptide with no labeling; NFL-SCR, the scrambled NFL-TBS.40-63 peptide (with the same amino acids as the NFL-peptide but in a different order); DMEM, dulbecco's modified eagle medium; SDS, sodium dodecyl sulfate; aCSF, artificial cerebrospinal fluid; TEM, transmission electron microscopy; XRS, x-ray scattering; UPLC, ultra-performance liquid chromatography system; CAC, critical aggregation concentration.

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<https://doi.org/10.1016/j.ijpx.2022.100128>

Received 29 August 2022; Accepted 30 August 2022

Available online 22 September 2022

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in GBM cells occurs by endocytosis and is likely mediated by several signaling pathways over-expressed in the active proliferative tumor cells (Lépinoux-Chambaud and Eyer, 2013). Taken together, NFL-peptide has been considered as promising therapeutic tool to target GBM.

Peptides can also self-assemble into supramolecular nanostructures that can serve as vehicles for targeted drug delivery (Habibi et al., 2016). Self-assembly is the process in which the peptide can self-associate via intermolecular interactions that can occur naturally in aqueous solutions (Dehsorkhi et al., 2014). The formation of peptide-based nanostructures appertains to a combination of various non-covalent interactions, including hydrogen-bonding (H-bonds), aromatic interactions (π - π stacking), hydrophobic forces, electrostatic and van der Waals interactions (Hartgerink et al., 2001). The different properties (aromatic/ aliphatic/ hydrophobic/ hydrophilic or neutral/ positively/ negatively charged) of amino acids forming the peptide are involved in its self-assembling ability (Dehsorkhi et al., 2014). The ionization degree of carboxylic acids and the interaction between multi amide groups in amino acids are also responsible for the formation and stabilization of peptide self-assembled nanostructures (Duan et al., 2011). Thus, the elucidation of peptide structure can highlight the potential ability of peptide to self-assemble and could help to understand the relationship between the molecular structures and self-assembly process (Valery et al., 2003). However, the physicochemical properties of peptides can be modified by various conditions such as environmental and solution parameters (pH, temperature and ionic strength) which can greatly influence their self-assembly mechanism (Ozkan et al., 2016).

Several types of nanoscale structures such as fibers, tubes and vesicles can be obtained from peptides through their self-assembly mechanism (Panda and Chauhan, 2014). Peptide nanostructures can be used to facilitate the delivery of various biomolecules into the cell (Lee et al., 2019; Avci et al., 2018). Self-assembling peptides can also be used as drugs themselves like the case of the Lanreotide peptide which has been approved by the FDA in the United States since 2007 and commercialized under the name Somatuline for acromegaly treatment (Valery et al., 2003). The ability of peptides to spontaneously organize into well-defined nanostructures is governed by their physicochemical properties and molecular structure (Lee et al., 2019). In aqueous solution, peptides start to aggregate at and above their critical aggregation concentration (CAC), and these aggregates then associate with each other to form self-assembled structures (Fung et al., 2003).

Here, we investigated the potential auto-assembly mechanism of the NFL-peptide by electronic microscopy observations. The critical aggregation concentration of the NFL-peptide was determined in this work by surface tension measurements. The structure of NFL-peptide was also defined by X-ray scattering (XRS). We further investigated the potential disassembly process of BIOT-NFL-nanofibers via a dialysis membrane paradigm. Moreover, the BIOT-NFL-peptide was incubated with gold nanoparticles to investigate the possible interaction between these particles and BIOT-NFL-nanofibers. Finally, the internalization of gold nanoparticles coupled to BIOT-NFL-nanofibers in F98 rat GBM cells was examined by transmission electron microscopy to inspect the possible use of peptide-nanofibers for the delivery of these particles to GBM for prospective thermotherapy purposes.

2. Materials and methods

2.1. Peptide synthesis

The NFL-TBS.40–63 peptide (NH_2 -YSSYPVSSSLSVRRSYSSSSGS-CONH₂) with a molecular mass of 2.4 kDa was synthesized by Polypeptide group (Strasbourg, France). Two different coupled peptides were supplied: NFL-peptide coupled to the biotin (BIOT-NFL) and NFL-peptide coupled to 5-Carboxy-fluorescein (FAM-NFL). Peptide labeling is necessary to follow the peptide in different biological assays, including fluorescence microscopy, flow cytometry, cell proliferation and survival assays. Two different types of labeling of the NFL-peptide

were used (biotin and fluorescein) and correspond to the peptides BIOT-NFL and FAM-NFL. An NFL-peptide with no labeling (nude-NFL) was also supplied.

A scrambled NFL-peptide (NFL-SCR), with the same amino acids as the NFL-peptide but in a different order was synthesized by Millegen (Toulouse, France). Scrambled peptides are made of the same amino acids but in the wrong order and therefore they are used to explore the impact of amino acid order on peptide properties (Berges et al., 2012b). Two labeled scramble-peptides were used: BIOT-NFL-SCR (BIOT-SPSVYSRSGSYASRSLSYSVSS-CONH₂) and FAM-NFL-SCR (FAM-SLGSPSSSVRASYSRRSYVYSSS-CONH₂). The purity of all peptides is $\geq 95\%$. All peptides were dissolved in Milli-Q water obtained from a MilliQ plus system (Millipore, Bilerica, USA) before use.

2.2. NFL-peptides preparation

The BIOT-, FAM-, nude-NFL-peptide as well as the scrambled-NFL-peptides (BIOT-NFL-SCR, and FAM-NFL-SCR) solutions were prepared. Peptide powders were dissolved in Milli-Q water at the concentration of 1 mmol/L at room temperature, and then a concentration of 0.2 mmol/L was prepared by diluting the initial solution (5 times in Milli-Q water) and stored at 4 °C. All NFL-peptide solutions obtained at the different concentrations were observed under transmission electron microscopy to investigate their potential self-assembly properties.

BIOT-NFL-peptide was also prepared in four different solutions. The two following solutions were supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France): D-glucose and Dulbecco's Modified Eagle Medium - high glucose (DMEM), while the two others were prepared in the laboratory: 2 mmol/L Sodium Dodecyl Sulfate (SDS; Sigma-Aldrich) and artificial cerebrospinal fluid (aCSF) (Davson, 1969). The BIOT-NFL-peptide powder was dissolved in sterile water at a concentration of 1 mmol/L, and then diluted 5 times by adding 2 μL of peptide at 1 mmol/L to 8 μL of adequate solution (SDS, DMEM, D-glucose, and aCSF) at room temperature. The four different solutions of BIOT-NFL-peptide at 0.2 mmol/L (Table 1) have a pH around 7 and have been stored at 4 °C for a long period of time (up to 30 days). Transmission electron microscopic observations of all samples were performed at different time points after preparation to evaluate the ability of BIOT-NFL to self-assemble in different solutions. All samples were observed at least in triplicate.

2.3. Transmission electron microscopy (TEM)

Samples observations were performed in the Microscopy Rennes Imaging Center platform (MRic TEM, Rennes, France). All peptide solutions were examined by transmission electron microscopy. Four microliter samples were deposited to glow-discharged electron microscope grids for 1 min and were negatively stained with 2% uranyl acetate for 10 s. The samples were observed using a 200 kV electron microscope (Tecnai G² T20 Sphera, FEI) equipped with a 4 k \times 4 k CMOS camera (model TemCam-F4160, TVIPS). Micrographs were acquired using the camera in binning mode 1.

Table 1

List of BIOT-NFL-peptide solutions.

Peptide	Solution	pH
BIOT-NFL (0.2 mmol/L)	2 mmol/L SDS	7
BIOT-NFL (0.2 mmol/L)	DMEM	7
BIOT-NFL (0.2 mmol/L)	4% D-glucose	7
BIOT-NFL (0.2 mmol/L)	aCSF	7

Four different solutions of BIOT-NFL at 0.2 mmol/L were prepared. The BIOT-NFL powder was dissolved in water at a concentration of 1 mmol/L, and then diluted 5 times by adding 2 μL of peptide at 1 mmol/L to 8 μL of adequate buffer (2 mmol/L SDS, DMEM, 4% D-glucose and aCSF) at room temperature. All solutions were stored at 4 °C for a long period of time (up to 30 days).

2.4. X-ray scattering (XRS)

For this experiment the NFL-peptide was prepared at a much higher concentration of 5 mmol/L in sterile water and then diluted twice in three different solutions: sterile water, 2.5% D-glucose and 1 mmol/L SDS buffer to obtain a 2.5 mmol/L final concentration of peptide. Peptide solutions were stored at 4 °C until analysis. The structural organization of peptide in the three different solutions was evaluated by X-ray scattering with a homemade setup. X-ray scattering results were collected with a Pilatus 300 K (Dectris, Switzerland), mounted on a micro source X-ray generator GeniX 3D (Xenocs, France) operating at 30 W. The monochromatic CuK_α radiation was $\lambda = 1.541 \text{ \AA}$. The results were recorded in a reciprocal space $q = (4\pi \cdot \sin\theta)/\lambda$ in a range of repetitive distances from 0.01 to 1.72 \AA^{-1} . The results were collected by a homemade program and analyzed by the Igor Pro 7.0 software (Wave metrics, US). All peptide samples were introduced into 1.5 mm quartz capillaries, then centrifuged and sealed with candle wax. For the analysis, they have been introduced in a capillary holder accommodating 19 capillaries at controlled temperature. Each capillary was probed at two y-positions to check the homogeneity of the sample. The analyses were carried out following a temperature ramp from 20 °C to 95 °C every 5 °C with an exposure time per point of 10 min. This experiment was performed on the BIOT-NFL as well as FAM-NFL to define the peptide structure.

2.5. Drop tensiometer study

Surface tension measurements were performed with a drop tensiometer device (Tracker, ITConcept, Longessaigne, France). These measurements can be made by analyzing the shape of a drop hanging from a needle. This approach is particularly suitable for very small volumes. In our case, the drop is a pendant drop of air (8 μL) formed using an microsyringe (Prolabo, Paris, France) into an optical glass cell (Hellma, France) containing either BIOT-NFL or FAM-NFL (with concentrations between 10^{-10} and 10^{-3} mol/L) prepared in water. The axial symmetric shape of the drop was analyzed using a video camera connected to a computer. Using Laplace's equation integration of the drop profile, the surface tension could be calculated and recorded (Mouzouvi et al., 2017). For each concentration, each surface tension was calculated once equilibrium was reached, and measurements were performed on three independent experiments.

2.6. Dialysis membrane study

In this experiment, Pur-A-Lyzer® dialysis tubes (10–250 μL) with molecular weight cut-off 6–8 kDa from Sigma-Aldrich were used. Prior to their use, the dialysis tubes were filled with Milli-Q water for 5 min as described by the manufacturer. The NFL-peptide was dissolved in Milli-Q water at the concentration of 1 mmol/L at room temperature (around 20 °C). Another concentration of peptide (0.2 mmol/L) was also prepared by diluting the initial concentration 5 times in water. After 48 h, 200 μL of each peptide concentration was deposited into the dialysis tube, and then the dialysis tube was placed in a beaker containing 10 mL of Milli-Q water under continuous stirring for 24 h. Several samples were taken from the outside of dialysis membrane at different time points (from 1 to 24 h) and the volume of each sample was replaced by an equal volume of Milli-Q water. The NFL-peptide concentrations in all samples were directly measured using Ultra Performance Liquid Chromatography system (Waters, Saint-Quentin-en-Yvelines, France) as described below, and these concentrations values were used to generate the NFL-peptide cumulative liberate profile.

Samples were also taken inside the dialysis tubes both at the beginning and at the end of dialysis experiment and were observed by transmission electron microscopy to investigate the density and the possible structure of peptide-nanofibers before and after the dialysis. TEM observations were performed at Service Commun d'Imageries et

d'Analyses Microscopiques (SCIAM; University of Angers, France), using a 120 kV electron microscope (Jeol, Japan) model JEM-1400, equipped with a Gatan SC1000 ORIUS® CCD camera (11 Megapixel) from USA. Briefly, a 2 μL of each sample were deposited on copper grids (150 mesh) and stained with 2% uranyl acetate for one minute, and then they were dried under room temperature before observation. This experiment was performed using the BIOT-, FAM- and nude-NFL-peptides with the same concentrations. This study was done in triplicate.

2.7. Quantification of NFL-peptides by ultra-performance liquid chromatography system (UPLC)

The peptide concentrations in all dialysis samples (outside the dialysis membrane) were directly measured using Ultra Performance Liquid Chromatography system (Waters) managed by Empower® 3 software (Waters). The column used in this method was the ACQUITY UPLC Peptide BEH C18 Column, 300 \AA , 1.7 μm , 2.1 mm \times 150 mm (Waters) at room temperature. Wavelength of the UV detector was set at 220 nm for BIOT- and nude-NFL-peptides. Fluorescence detector (excitation wavelength: 486 nm - emission wavelength: 519 nm) was used for FAM-NFL-peptide. The analysis was performed using the following mobile phases (solvent A: water-ACN-TFA 95:5:0.1, and solvent B: 0.1% TFA in ACN). The runtime of the analysis was 30 min, the flow rate was maintained at 0.28 mL/min and the injection volume was 10 μL . Samples temperature was fixed at 4 °C. A symmetric peak of BIOT-NFL (Fig. S1) and the two peaks for other peptides (FAM- and nude-NFL) was obtained. The retention time of BIOT-, FAM- and nude-NFL-peptides was 23.6, 26.2 and 21.5 min respectively. A calibration curve of NFL-peptide diluted in Milli-Q water (from 2 to 12.5 $\mu\text{mol/L}$) was obtained by quantifying the area under the peak. All dialysis samples (collected from 1 to 24 h of dialysis in polypropylene vials without filtration) were directly measured by UPLC using this method. The area under the peptide peak was proportional to the NFL-peptide concentration outside the dialysis membrane.

2.8. Coupling between the BIOT-NFL-peptide-nanofibers and gold nanoparticles

6 nm gold nanoparticles coupled with streptavidin (106.099) have been purchased from Aurion (PD Wageningen, the Netherlands). An incubation of 24 h at room temperature with gentle stirring between the BIOT-NFL at 1 mmol/L and gold nanoparticles at 200 mg/mL was realized. Samples observations were performed with a transmission electronic microscope in the Microscopy Rennes Imaging Center platform (MRic TEM, Rennes, France) as previously described.

2.9. Glioblastoma cell treatment with gold nanoparticles coupled with the BIOT-NFL-peptide-nanofibers

F98 rat 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 antibiotics (100 \times streptomycin/penicillin; BioWest, Nuaille, France) and 1% of non-essential amino acids (Sigma-Aldrich). Cells were seeded in 6-well plates at 400,000 cells per well and incubated for 24 h at 37 °C and 5% CO_2 . Then, gold nanoparticles without or with the BIOT-NFL-nanofibers were incubated for 72 h. After incubation, cells were washed with 0.1 mol/L phosphate buffer at pH 7.4 and fixed with a solution of 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, overnight at 4 °C. The next day, the fixator was removed, and cells were rinsed with 0.1 mol/L phosphate buffer. Then, cells were rinsed with 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 15 min in 50° ethanol, 15 min in 70° ethanol, 15 min in 95° ethanol and 3 times 30 min in 100° ethanol. The cells were placed in a solution of 50% 100° ethanol and 50% Epon resin mixture (v/v)

overnight. The next day, the Epon was removed and replaced by a pure Epon bath for 4 h, then this bath was replaced by another pure Epon bath 24 h at 37 °C, then 24 h at 45 °C and 72 h at 60 °C. When the resin has polymerized 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 model 832 (Gatan) 4 k CCD camera.

3. Results

3.1. Analysis of NFL-peptides self-assembly

Labeling of the NFL-peptide is necessary to follow the peptide or its effects in different bioassays. For this purpose, we use mainly two different labels (biotin and fluorescein) depending on the appropriate assay. For example, the FAM-NFL-peptide is used for the flow cytometry assay (Fluorescence-Activated Cell Sorting) or the immunofluorescence assay to study the cellular uptake of the NFL-peptide, while the BIOT-NFL-peptide is used for the possible effect on cell cycle, cell viability (Trypan blue exclusion) or CyQUANT cell proliferation assay. In

addition, either labeled peptide is used depending on the cellular process to be monitored, such as the microtubule or the actin cytoskeleton, the mitochondria network, or the cellular uptake of nanocapsules filled with the fluorochrome DiD. These different labels have been used for >15 years without knowing if they could affect the ultrastructure of the NFL-peptide and its organization or activity. The aim of this study is to know if the markers used could affect these different NFL-peptides.

The different types of NFL-peptides (BIOT-, FAM-, or nude-NFL) as well as the BIOT-NFL-SCR and FAM-NFL-SCR dissolved in water have been examined by using transmission electron microscopy. Interestingly, TEM micrographs showed that the NFL-peptide coupled to the biotin (BIOT-NFL) can spontaneously form peptide-nanofibers in water even at a concentration as low as 0.2 mmol/L (Fig. 1A). Well organized BIOT-NFL-nanofibers with a diameter of about 5 nm were obtained. The length (several micrometers) and density of BIOT-NFL-nanofibers increased as the concentration increased, with a very dense population of nanofibers observed at the high concentration (1 mmol/L), as shown in Fig. S2.

We also investigated the possible formation of peptide-nanofibers from other types of NFL-peptides (FAM-, nude-, or scrambled-NFL) that we use in our tests, to study the impact of labeling or not of NFL-peptide or changing its order of amino acids on peptide properties. Electron microscopy observations showed that the scrambled peptides,

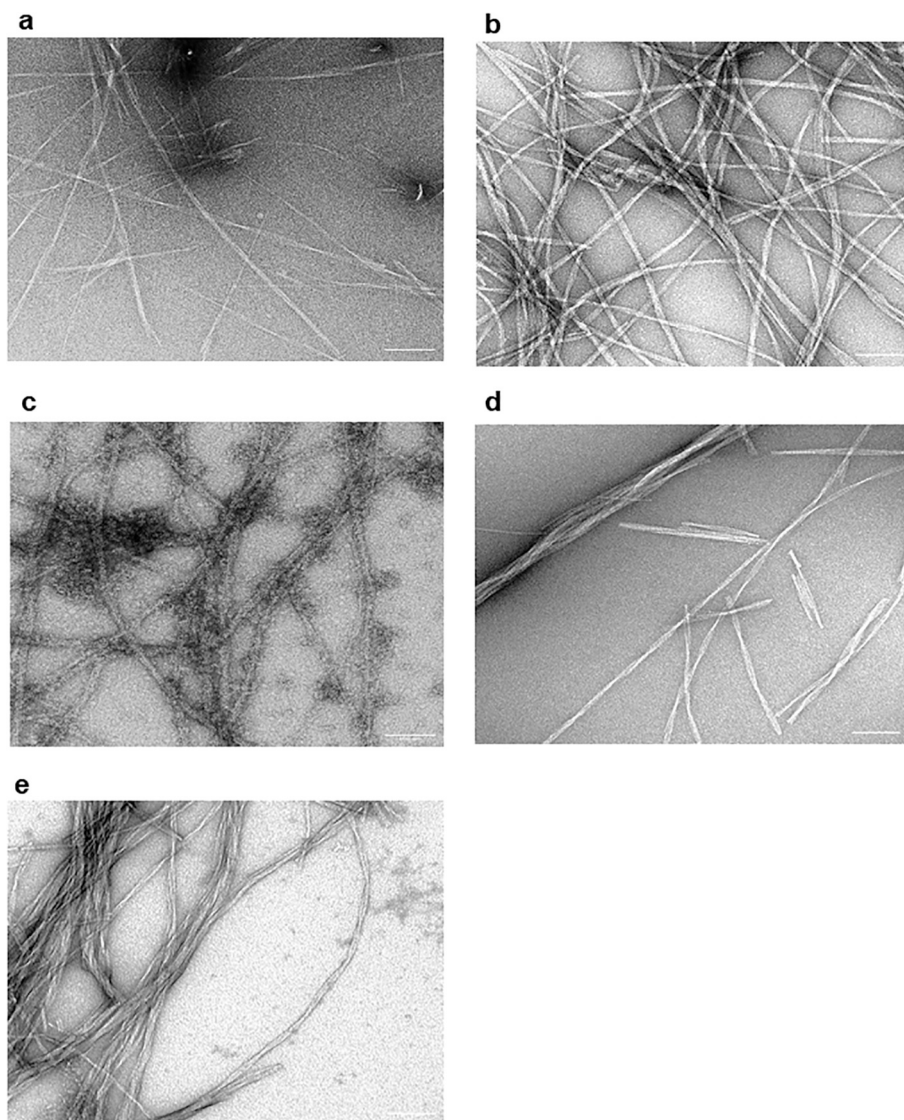


Fig. 1. Analysis of NFL-peptide self-assembly by transmission electron microscope.

The BIOT-NFL-peptide was dissolved in Milli-Q water at a concentration of 1 mmol/L and then diluted 5 times in water to obtain a final concentration of 0.2 mmol/L. Four different solutions (SDS, DMEM, 4% D-glucose and aCSF) were prepared similarly for the BIOT-NFL at 0.2 mmol/L. All different solutions were stored at 4 °C. A 4 µL of each sample was deposited on glow-discharged electron microscope grids and negatively stained with 2% uranyl acetate for 10 s, and then they were dried under room temperature before TEM observation. **a)** BIOT-NFL at 0.2 mmol/L in water. **b)** BIOT-NFL at 0.2 mmol/L in SDS. **c)** BIOT-NFL at 0.2 mmol/L in DMEM. **d)** BIOT-NFL at 0.2 mmol/L in 4% D-glucose. **e)** BIOT-NFL at 0.2 mmol/L in aCSF. Bar 100 nm.

that have the same amino acids as the NFL-peptide but in a different order, formed peptide-nanofibers with slightly different structure either coupled to biotin (BIOT-NFL-SCR) or 5-Carboxy-fluorescein (FAM-NFL-SCR) (Fig. S3A-B, respectively). On the opposite, very few and short fibers were observed from the non-coupled NFL-peptide (nude-NFL), or the NFL-peptide coupled to the 5-Carboxy-fluorescein (FAM-NFL); a few stick-shaped filaments were observed in the two cases (Fig.— S3C-D, respectively). These observations clearly indicate that the conformation of these NFL-peptides in solution is different.

3.2. Peptide-nanofibers formed from BIOT-NFL in different solutions

The solution components have also a significant effect on the possibility of self-assembly. It can cause a huge alteration in the morphology of nanofiber structure (Cui et al., 2010). In this regard, we investigated the effect of aqueous buffer on BIOT-NFL self-assembly. Four different solutions of BIOT-NFL at a concentration of 0.2 mmol/L and pH 7 were prepared (Table 1) as previously described. Interestingly, BIOT-NFL was able to form peptide-nanofibers with about 5 nm in diameter and several micrometers in length in all studied solutions. However, the arrangement and the density of BIOT-NFL-nanofibers were different according

to solution components. A very dense network of thin and long nanofibers formed from BIOT-NFL was observed in SDS buffer (Fig. 1B). The BIOT-NFL was also able to form nanofibers in the cell culture medium (DMEM) as shown in Fig. 1C. In the case of 4% D-glucose, individual short nanofibers of BIOT-NFL were observed (Fig. 1D). As seen in Fig. 1E, nanofibers arranged in a parallel manner were obtained from the BIOT-NFL when it is prepared in the aCSF buffer (artificial cerebrospinal fluid buffer).

The stability of these nanofibers was also investigated over time. Nanofibers obtained from BIOT-NFL were found to be stable after long period of peptide preparation in all studied cases (data not shown). The present findings indicate that the BIOT-NFL can organize into well-defined nanofibers (diameter ~ 5 nm) in different buffers and these nanofibers are stable overtime (up to 30 days).

3.3. NFL-TBS.40–63 peptide structure determination by X-ray scattering

In an attempt to understand the relationship between the structure of peptide and its ability or not to form peptide-nanofibers, the two peptides (BIOT-, and FAM-NFL) were analyzed by X-ray scattering in different solutions. Sterile water was used in this experiment as a

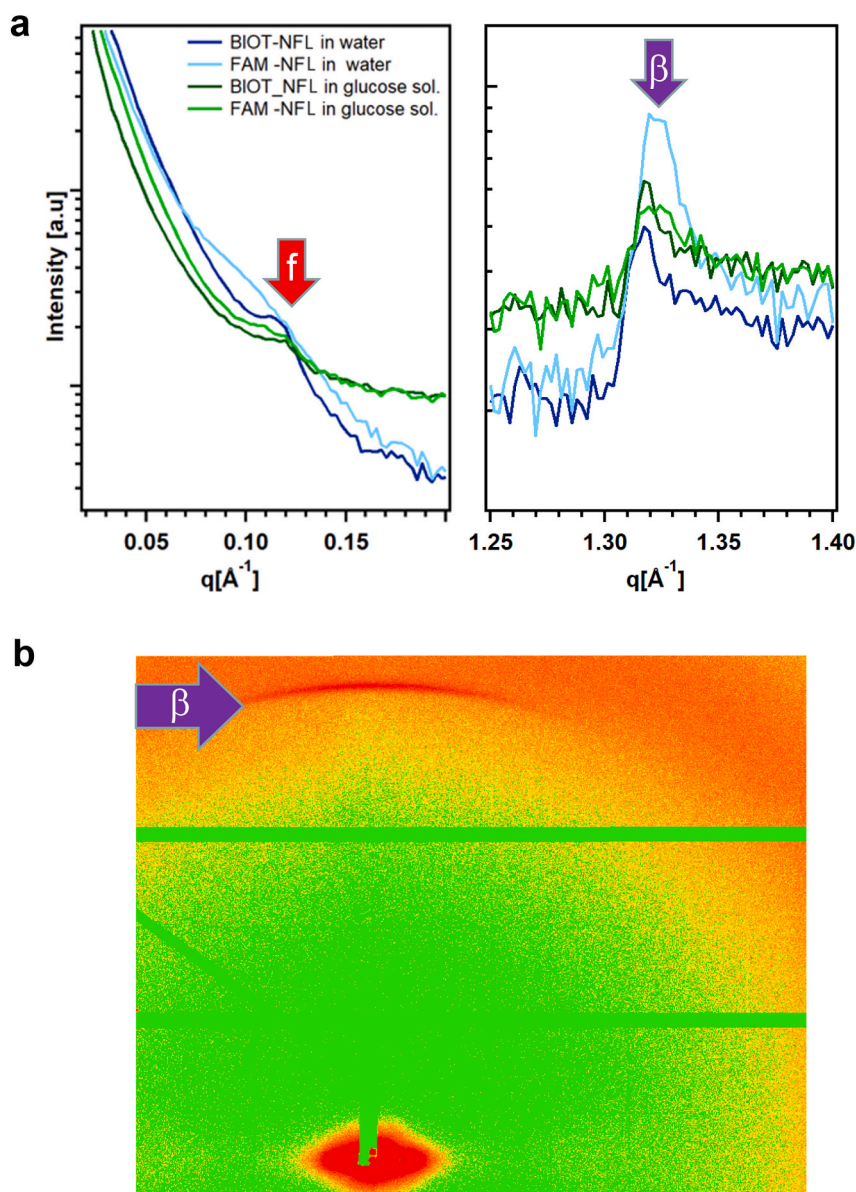


Fig. 2. X-ray scattering of NFL-peptide. **a)** The BIOT- and FAM-NFL-peptides were prepared at the concentration of 2.5 mmol/L in two different solutions (sterile water and 2.5% D-glucose). Dark blue: BIOT-NFL at 2.5 mmol/L in water. Light blue: FAM-NFL at 2.5 mmol/L in water. Dark green: BIOT-NFL at 2.5 mmol/L in 2.5% D-glucose. Light green: FAM-NFL at 2.5 mmol/L in 2.5% D-glucose. **b)** Oriented Pattern obtained by slow drying of FAM-NFL-peptide in water. Arrows indicate β -sheet X-ray-scattering.

reference to evaluate the effect of buffers on the peptide structure. Fig. 2 shows the X-ray scattering results of BIOT- and FAM-NFL-peptides in 2.5% D-glucose and sterile water. The observation of $1.31\text{--}1.32\text{ \AA}^{-1}$ peaks in all spectra (Fig. 2A) indicate that the two types of NFL-peptides (BIOT- and FAM-NFL) adopt a β -sheet structure in the studied solutions

at physiological pH (Sunde et al., 1997). Similar results were obtained in the SDS buffer (data not shown). Lateral assembly was also observed at Small Angle at 0.1 \AA^{-1} and can be attributed to lateral packing within the fiber (Fitzpatrick et al., 2013). Oriented samples were obtained by drying (Fig. 2B) and confirm the X-ray scattering analysis of solution

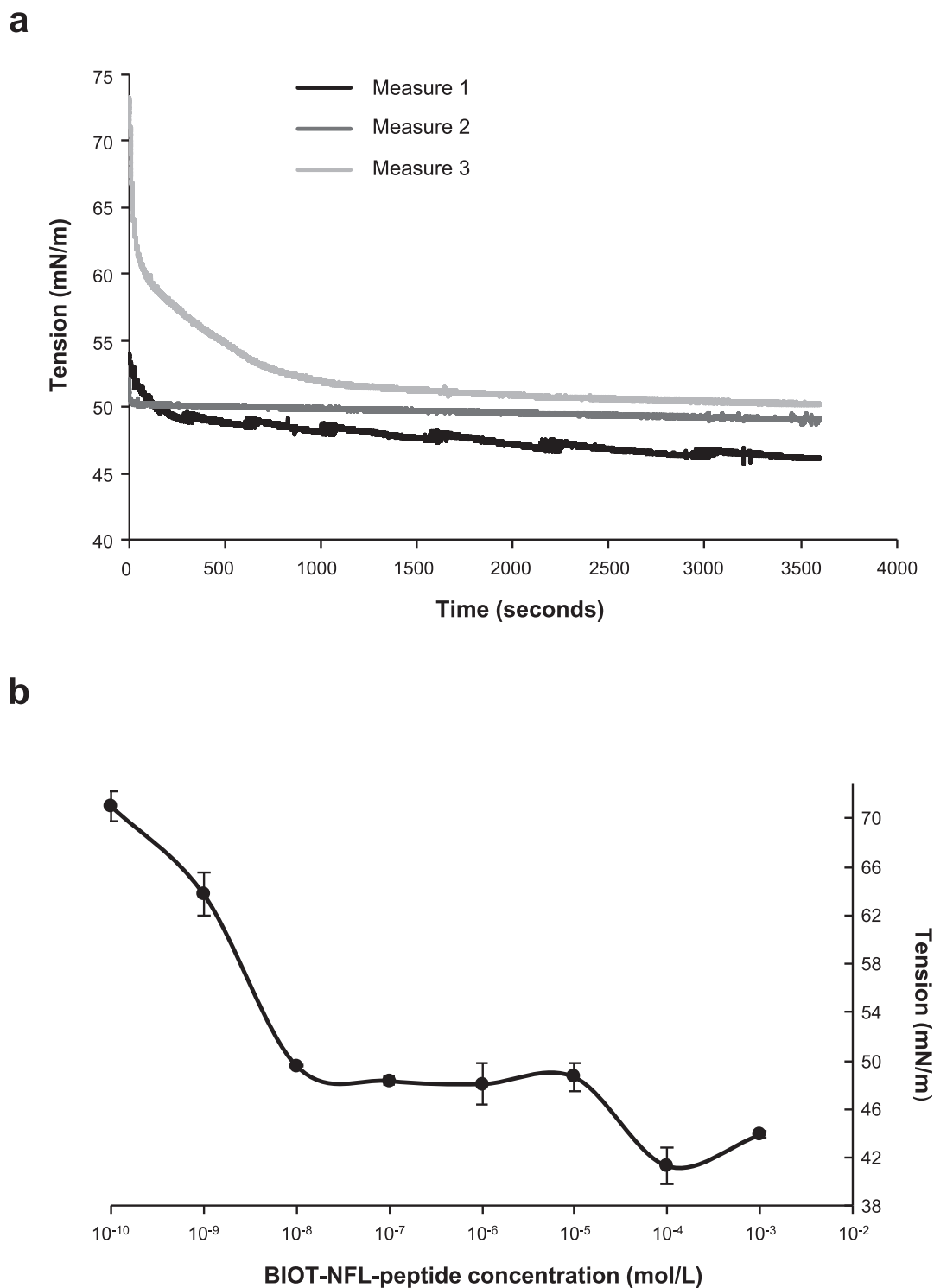


Fig. 3. Analysis with a drop tensiometer of surface tension between the BIOT-NFL-peptide and the air. Measurements were realized between an 8 μL pendant drop of air and a glass cell containing the BIOT-NFL (between 10^{-10} and 10^{-3} mol/L). For each concentration, surface tension was calculated to obtain equilibrium. Measurements were performed on three independent droplets. **a)** Measures of surface tension of the BIOT-NFL at 10^{-5} mol/L. **b)** Determination of the critical aggregation concentration of the BIOT-NFL. Experiments were performed at least in triplicate. Data are represented as the mean \pm SEM.

samples. The fiber consequently exhibits amyloid-like organization.

3.4. Interfacial properties of the NFL-peptide

Surface tension between the NFL-peptide and air were realized with a drop tensiometer. For each peptide (BIOT-, or FAM-NFL), surface

tension with air was measured three times for eight concentrations. Then, for each concentration we were able to draw a graph of the surface tension over time. Using this graph, we were able to determine the surface tension at equilibrium. Fig. 3A and 4A represent, respectively, measures of surface tension of the BIOT-NFL or of the FAM-NFL at 10^{-5} mol/L. Then, we can draw a graph representing all surface tensions at

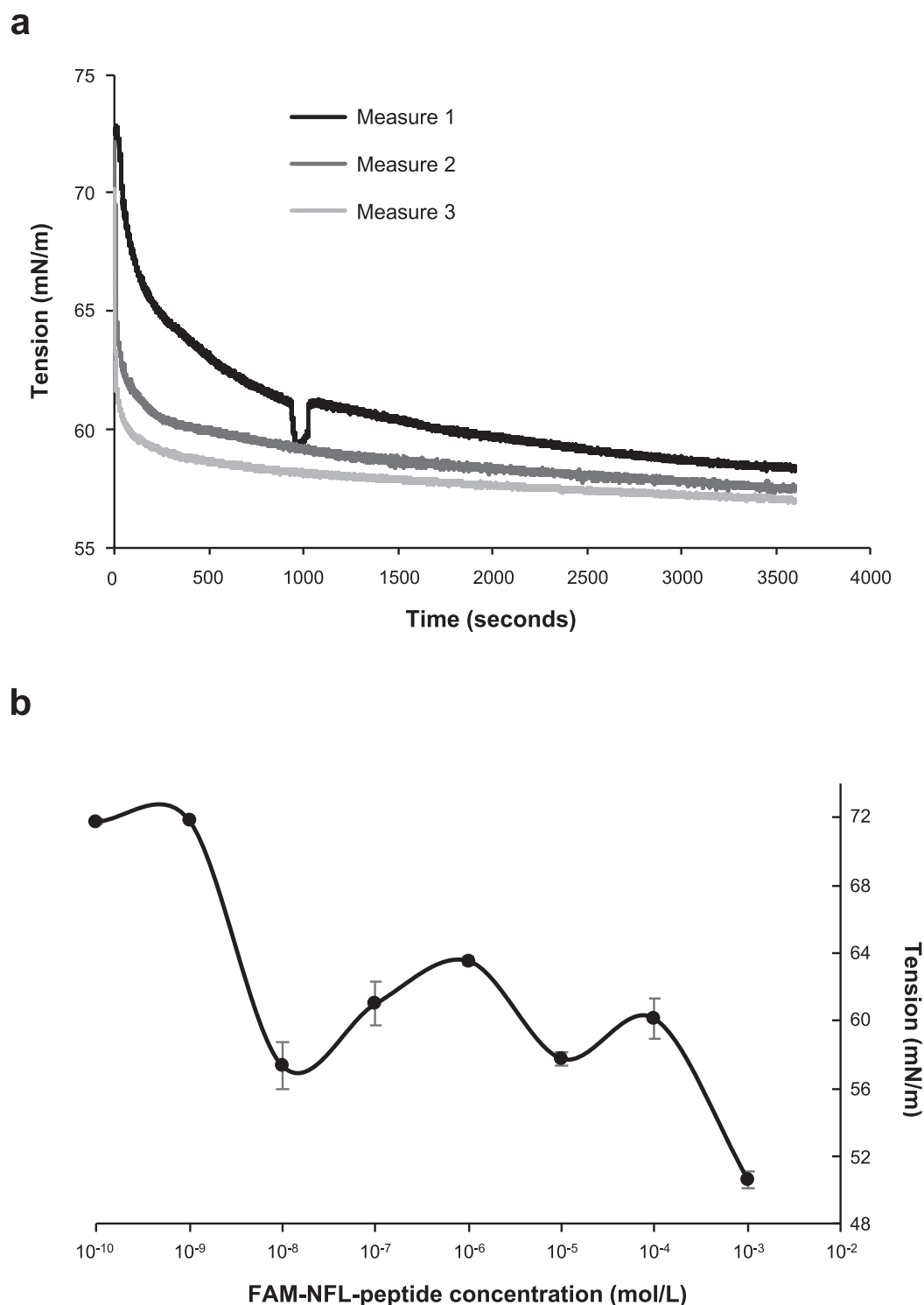


Fig. 4. Analysis with a drop tensiometer of surface tension between the FAM-NFL-peptide and the air. Measurements were realized between an 8 μ L pendant drop of air and a glass cell containing the FAM-NFL (between 10^{-10} and 10^{-3} mol/L). For each concentration, surface tension was calculated to obtain equilibrium. Measurements were performed on three independent droplets. **a)** Measures of surface tension of the FAM-NFL at 10^{-5} mol/L. **b)** Determination of the critical aggregation concentration of FAM-NFL. Experiments were performed at least in triplicate. Data are represented as the mean \pm SEM.

equilibrium as a function of the peptide concentration logarithm. With this graph, we can determine the critical aggregation concentration (CAC), and this CAC is based on amphiphilic structures. The knowledge of the CAC is fundamental to study the self-aggregation of amphiphilic molecules in solution. For the BIOT-NFL at 10^{-10} mol/L, the surface tension is near to that of water tension (which is 72.8 mN/m at 20 °C) (Pallas and Harrison, 1990), thus the solution contains more water molecules than peptide. Then, the surface tension decreases and becomes stable (around of 50 mN/m) between the concentrations of 10^{-8} and 10^{-5} mol/L, and at 10^{-4} mol/L the surface tension reaches its minimum (41 mN/m) (Fig. 3B). This concentration (10^{-4} mol/L) reflects the lowest BIOT-NFL concentration that generates aggregates. Same experiments were realized with the FAM-NFL; at 10^{-10} mol/L the surface tension is near to that of water tension. Then, the surface tension between the FAM-NFL and air decreases then oscillates between 63 and 57 mN/m (Fig. 4B).

3.5. Diffusion of NFL-peptides using a dialysis paradigm

The purpose of this study was to investigate the diffusion of NFL-peptide via a dialysis membrane under the conditions used in dialysis experiment. An increased amount of BIOT-NFL was detected over time outside of the dialysis membrane, suggesting that the fibers are disassembling under these conditions, and the free peptides exit the dialysis tube. Only the results of the high concentration (1 mmol/L) of BIOT-NFL were presented in Fig. 5. To further confirm such a possibility, the structure of the nanofibers formed by the BIOT-NFL inside the dialysis tube was also studied by transmission electron microscopy both at the beginning and at the end of the dialysis period. TEM observations show a clear alteration of the density and the length of BIOT-NFL-nanofibers

following the dialysis process. The highly dense network of long nanofibers observed inside the dialysis tube at the beginning of experiment (Fig. 5A), became noticeably less dense after 24 h of dialysis, and much shorter nanofibers were observed as seen in Fig. 5B. However, many long fibers were still observed. Together, UPLC measurements (Fig. 5C) and TEM observations suggest that a peptide monomer can disengage from the auto-assembled peptides-nanofiber (composed of hundreds or thousands peptide molecules) under these conditions and pass through the dialysis membrane (BIOT-NFL-peptide = 2.7 kDa versus dialysis membrane cut-off = 6–8 kDa) during dialysis. In fact, the dilution induced by the dialysis process decreases the concentration of peptide inside the dialysis tube, which in turn can change the arrangement of monomeric peptides in the solution and change the form of peptide-nanofibers (Yan et al., 2008).

The same experiment was performed using the FAM- and the nude-NFL at the same concentrations. As we demonstrated previously, much less, and short nanofibers were observed from these two peptides. UPLC measurements also showed a cumulative release of FAM- and nude-NFL-peptides in all concentrations. Fig. S4 represents the diffusion of the FAM- and the nude-NFL outside from the dialysis tube at 1 mmol/L. No major difference was observed between the diffusion of these two peptides (FAM- and nude-NFL-peptides existing mainly as individual peptide molecules) and the diffusion of BIOT-NFL-peptide assembled into nanofibers (Fig. 5C) at the same concentration. These results suggest that peptide-nanofibers obtained from BIOT-NFL at 1 mmol/L can disassemble during dialysis, due to dilution effect, into free peptide molecules that can pass through the dialysis membrane as an individual peptide.

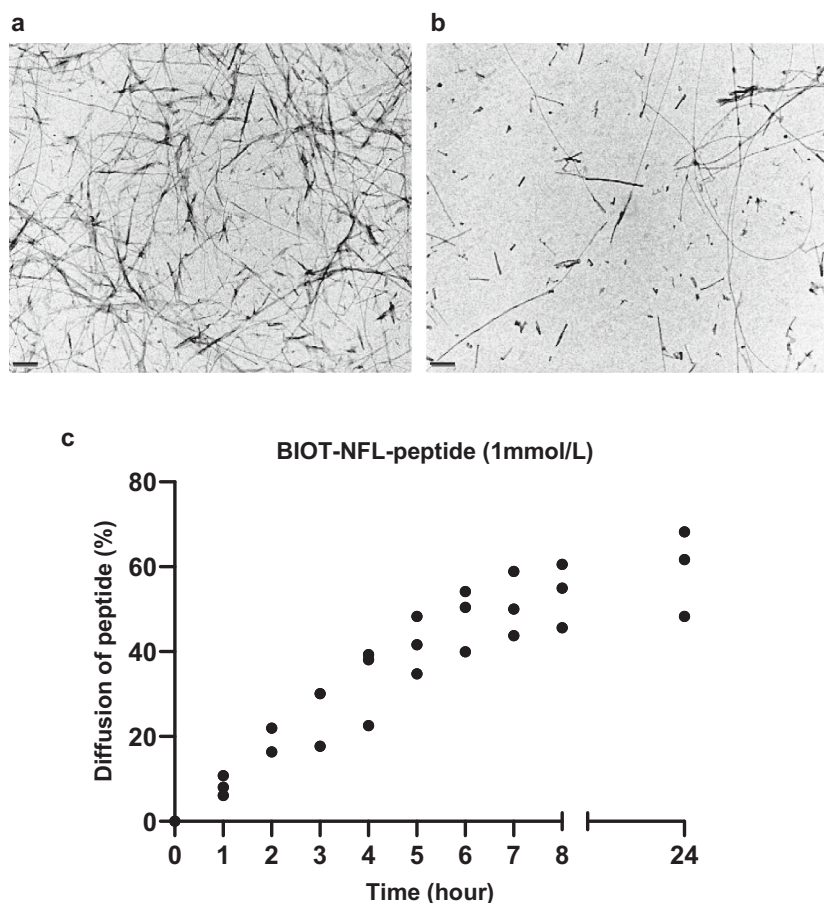


Fig. 5. Diffusion of BIOT-NFL-peptide during dialysis. A 6–8 kD MWCO Pur-A-Lyzer dialysis tube was used to evaluate the peptide-nanofibers disassembly. The BIOT-NFL was prepared in Milli-Q water at 1 mmol/L. After 48 h, 200 μ L of peptide solution was deposited into dialysis tube and then placed in a beaker containing 10 mL of Milli-Q water under continues stirring. Several samples were taken from the outside of dialysis membrane at different time points (until 24 h). The figure represents TEM micrographs of BIOT-NFL (1 mmol/L) at the onset (a) and the end (b) of dialysis, Bar 200 nm, with the UPLC quantification (c) of BIOT-NFL at the same concentration outside the dialysis tube.

3.6. Interaction between the nanofibers formed by the BIOT-NFL-peptide and gold nanoparticles and their internalization in rat glioblastoma cells

6 nm gold nanoparticles functionalized with streptavidin at 200 mg/mL were incubated with the BIOT-NFL at 1 mmol/L for 24 h. Then, the samples were observed by transmission electron microscopy. The results showed that the nanofibers formed by the BIOT-NFL were decorated by gold nanoparticles (Fig. 6A).

Then, we evaluated whether gold nanoparticles can penetrate in F98 rat GBM cells when functionalized with the BIOT-NFL. F98 cells were treated for 72 h with gold nanoparticles alone or gold nanoparticles coupled with the BIOT-NFL-nanofibers. Then, cells were fixed, included, and sectioned with a UC7 ultramicrotome and observed with a transmission electron microscope. The results showed that when cells were treated with gold nanoparticles alone, few gold nanoparticles are present into F98 cells, and mostly in cellular vacuoles (Fig. 6B). When the BIOT-NFL-nanofibers decorated by gold nanoparticles were incubated with the F98 cells in the same conditions, much more gold nanoparticles are present into cells (Fig. 6C). These results show the importance of the presence of the BIOT-NFL in helping the entry of gold nanoparticles into F98 cells. Experiments were realized with a treatment of 24 h or 48 h, and similar results were observed (data not shown).

4. Discussion

Self-assembly of peptides facilitates the formation of nanostructures that play an important role in translocating therapeutics into the cell (Pujals et al., 2006). Self-assembling peptides can also be used as drugs themselves (Valery et al., 2003). NFL-TBS₄₀₋₆₃ peptide is a cell-penetrating peptide that can enter selectively in several GBM cell lines mainly via endocytosis pathways (temperature and energy-dependent), where it destroys their microtubule network and provokes apoptosis without damaging the other healthy cells of the nervous system (Berges et al., 2012a; Lépinoux-Chambaud and Eyer, 2013; Balzeau et al., 2013).

Different NFL-peptides have been used in our previous works: labeling NFL-peptides were used to track the peptide in different biological tests, and scrambled NFL-peptides were used to study the importance of amino acids order on the biological function of this peptide (Berges et al., 2012b). Here, our study aimed to explore the possible effects of different labels on the conformation of the different NFL-peptides in solution or their possible auto-assembly.

Interestingly, in this work we demonstrated the ability of the Biotinylated-NFL-peptide (BIOT-NFL) to spontaneously self-assemble into nanofibers approximately 5 nm in diameter and several micrometers in length in aqueous solutions. Well organized nanofibers were spontaneously obtained from BIOT-NFL in all studied concentrations. As seen in Fig. 1A, BIOT-NFL-nanofibers were observed by transmission electron microscopy at a concentration of 0.2 mmol/L in water. More

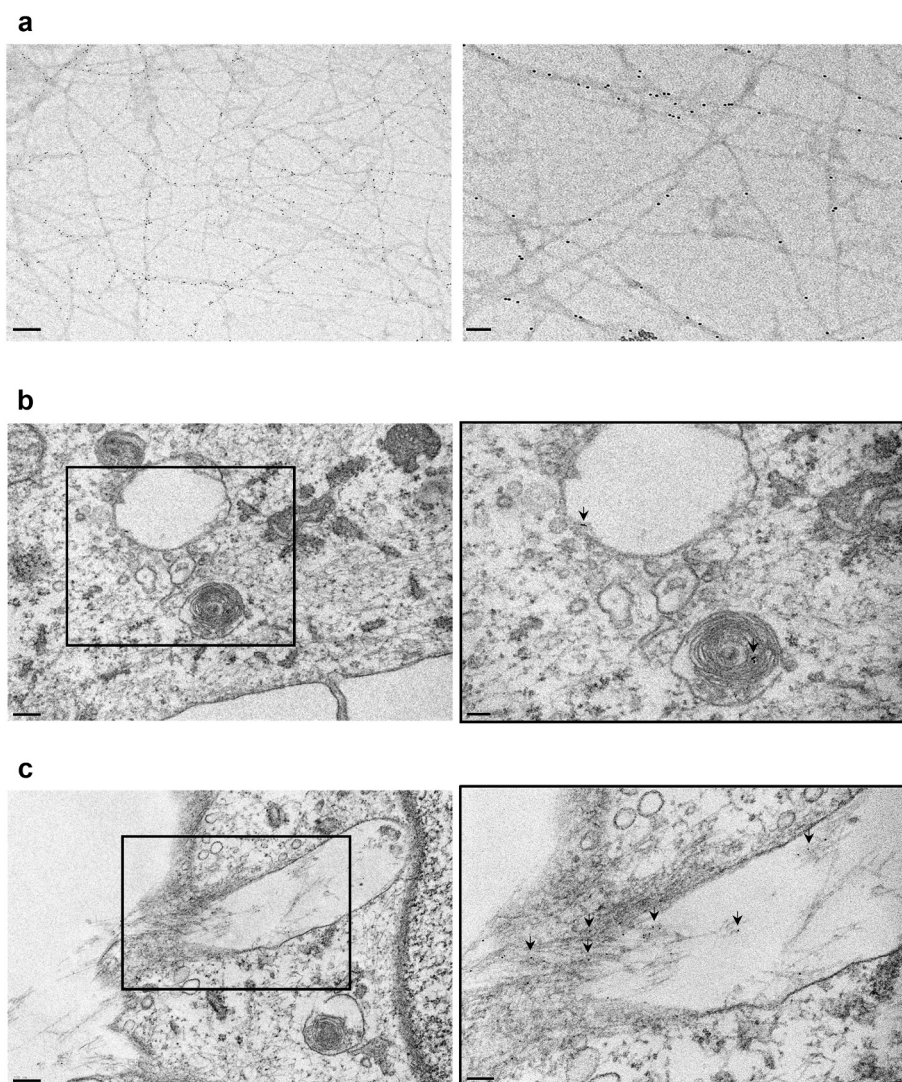


Fig. 6. Internalization of gold nanoparticles-streptavidin coupled with the BIOT-NFL-peptide into F98 cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a) 6 nm gold nanoparticles coupled streptavidin at 200 mg/mL were incubated with BIOT-NFL at 1 mmol/L for 24 h. Observations were performed with a transmission electronic microscope. The nanofibers formed by the BIOT-NFL were decorated with gold nanoparticles. Bar (at left) = 200 nm. Bar (at right) = 50 nm. **b)** F98 cells were incubated with 6 nm gold nanoparticles-streptavidin at 200 mg/mL for 72 h. Cells were fixed and were observed with a transmission electronic microscope. Few gold nanoparticles were internalized in the cells, and mostly in cellular vacuoles. Bar (at left) = 200 nm. Bar (at right) = 100 nm. **c)** F98 cells were incubated with 6 nm gold nanoparticles-streptavidin at 200 mg/mL coupled with the BIOT-NFL at 1 mmol/L for 72 h. Cells were fixed and were observed with a transmission electronic microscope. Nanofibers of BIOT-NFL decorated with gold nanoparticles were internalized in F98 cells, and much more gold nanoparticles were observed in the cells when the BIOT-NFL is present. Bar (at left) = 200 nm. Bar (at right) = 100 nm. The arrows in the figures show the gold nanoparticles.

nanofibers were found with increasing concentration as shown in Fig. S2, when the BIOT-NFL was observed at 1 mmol/L. Thus, the formation of these nanofibers from the self-assembly of BIOT-NFL-peptide monomers seems to be concentration dependent (Fig. S5) (Ghosh and Fernández, 2020; Sivagnanam et al., 2019). This is probably attributed to the sufficient free energy association gained by intermolecular interactions at high concentrations of BIOT-NFL (Yan et al., 2008). The dense network of nanofibers obtained from the BIOT-NFL at 1 mmol/L may explain the capacity of this peptide to form a viscous gel at higher concentrations (data not shown). As described in the literature, self-assembling peptide-nanofiber hydrogels have excellent mechanical characteristics and favorable rheological properties, so they have been widely studied in drug delivery applications (Sun and Wu, 2020; Guo et al., 2020). Thus, the formation of peptide-nanofibers from the BIOT-NFL-peptide is potentially beneficial for delivery purposes by different modes of administrations. The possibility of producing sterile BIOT-NFL-peptide-based gels with adequate fluidity to exit through a syringe needle to be placed in the tumor cavity after tumor excision is a very promising prospect but still requires a lot of work and is not the main focus of this article.

The other types of NFL-peptides were also observed by electron microscopy to examine their configuration in solution. Peptide-nanofibers with slightly different ultra-structure were observed in the case of BIOT-NFL-SCR (Fig. S3A) as well as the FAM-NFL-SCR (Fig. S3B) that have the same amino acids as the NFL-peptide but in a different order, while very few and short fibers were obtained from the nude-NFL (NFL-peptide with no labeling) or the FAM-NFL (NFL-peptide coupled to 5-Carboxy-fluorescein) (Fig. S3C-D, respectively). The present findings suggest that the configuration of NFL-peptides in solution seems to be different.

In fact, the self-assembly mechanism of NFL-peptide appears to correlate with different factors. The coupling of NFL-peptide may induce structural modifications on the peptide terminal domain that may facilitate (or not) the auto-assembly of peptide molecules, which may explain the difference of behavior between the NFL-peptide coupled to biotin (BIOT-NFL) and the non-coupled NFL-peptide (nude-NFL). The order of amino acids has also changed the ability of this peptide to self-assemble under the same conditions (FAM-NFL vs. FAM-NFL-SCR). Interestingly, it was previously shown that these scramble peptides do not enter in glioblastoma cells or much less (Berges et al., 2012b), indicating that the organization into nanofibers is not a prerequisite for cell penetration of this peptide.

The different self-assembly properties of variant NFL-peptides can probably be explained by the modification made on this peptide (changing the arrangement of amino acids and coupling or not the peptide-terminal). These modifications may induce alteration in peptide structure, and hence the capacity of peptide to self-assemble. In the nature, it is known that different kinds of proteins, DNAs, and RNAs with distinct biological structures and function have been generated with only twenty kinds of amino acids and quite a few kinds of nucleotides, this phenomenon illustrates the effects of composition, and sequence of molecules on their self-assembly behaviors (Zhang et al., 2019). Thus, the different self-assembly behavior of NFL-peptides may be impacted by peptide modifications. In this study, the BIOT-NFL-peptide demonstrated its ability to arrange into long and well-organized nanofibers in solution. As described in the literature, the presence of biotin onto the peptide improves its chemical and steric configuration, and consequently active targeting on cancer cells (Arib et al., 2022). Our hypothesis is that as biotin has a role in the chemical and steric peptide configuration, it may also participate in the arrangement and auto-association of peptide monomers in solution to form the well-organized nanofibers. However, further studies including molecular modelling (Berges et al., 2012b) as well as circular dichroism and NMR analysis are needed to better understand the self-assembly process and the variation between the different NFL-peptides.

BIOT-NFL-peptide was also able to form nanofibers in buffers that

can mimic the environment of this peptide in its biological tests as well as in the organism (Fig. 1). These nanofibers were stable up to 30 days (data not shown), but the density and the ultrastructure of the fibers can differ according to solution (Cui et al., 2010). A very dense network of nanofibers was obtained from BIOT-NFL in the SDS (Fig. 1B), an anionic surfactant that is commonly used to mimic the lipid environment of a cell membrane (Xu and Keiderling, 2008), which reveals that this peptide can form nanofibers around cells. The formation of BIOT-NFL-nanofibers in the cell culture medium used for the in vitro models of GBM cells (DMEM), also confirms the peptide ability to organize into nanofibers when in contact with cells in culture (Fig. 1C). These observations suggest that this peptide could be organized into nanofibers around cells. Indeed, nanofibers formed from BIOT-NFL were observed in 4% D-glucose (Fig. 1D), which is important by the fact that this peptide might form nanofibers in an intravenous solution that is usually used for the administration of peptide in animal models. Another potential route of NFL-peptide administration is the placement of a peptide-loaded implant in the tumor cavity during the surgical resection of glioma to provide a sustained release of this peptide. The capacity of BIOT-NFL to organize into nanofibers in the aCSF (Fig. 1E), indicates that this peptide might be also in the form of nanofibers when it is injected locally in the brain around tumor (Berges et al., 2012a).

The formation of self-assembled peptide nanofibers depends on several factors, including the peptide structure (Li et al., 2022). As demonstrated by XRS analysis in Fig. 2, NFL-peptide adopts a β -sheet structure in different solutions (water and 2.5% D-glucose). β -Sheets are the most common natural motifs that can be used in driving the self-assembly of peptides by facilitating the intermolecular hydrogen bonding between sheet-like structure regions of neighboring peptide molecules (Habibi et al., 2016; Missirlis et al., 2011). Several examples have been reported in the literature regarding the design of a class of peptide amphiphiles by incorporating a hydrocarbon tail on a β -sheet forming peptide to obtain self-assembled cylindrical nanofibers under physiological conditions (Zhang et al., 2013), i.e. N-terminal palmitic acid was added to form the hydrophobic peptide amphiphilic tail of β -sheet forming amino acids to form self-assembled nanofibers (Matson et al., 2012).

It appears that the structure of BIOT-NFL along with the alternating hydrophilic and hydrophobic amino acids in its sequence, which provide an amphiphilic property, can facilitate the intermolecular interaction and lead to the self-assembly of β -sheets (Fishwick et al., 2003). The presence of hydrophobic amino acids such as aromatic and aliphatic amino acids leads to hydrophobic interactions and aggregation through π - π stacking, whereas the hydrophilic amino acids are mainly responsible of electrostatic interactions or hydrogen bonding (Leite et al., 2015).

The various non-covalent interactions (e.g., hydrogen bonding, hydrophobic and/or aromatic interactions, and electrostatic interactions) are known to be primarily responsible for peptide self-assembly in aqueous solutions, and each of them has a specific role in directing self-assembled nanostructures (Zhao et al., 2016). All these interactions are key contributors of peptide self-assembly (Toksoz et al., 2010). Nevertheless, the underlying molecular mechanisms for the self-assembled peptide-nanofibers are still unclear to us because of the different behavior between the peptide coupled to biotin (BIOT-NFL) and other NFL-peptides.

The critical aggregation concentration (CAC) is another important parameter to describe the self-assembly ability of a molecule. According to the results obtained with the drop tensiometer (Saulnier et al., 2001), the critical aggregation concentration (CAC) of the BIOT-NFL is 10^{-4} mol/L (Fig. 3B). At this concentration, there are the most interactions between the peptide solution and the air. This result revealed the amphiphilic property of the BIOT-NFL and its ability to aggregate in solution at concentrations above the CAC value. The continuous peptide aggregates can then join to create long nanofiber. For the FAM-NFL, we could not determine its CAC because the surface tension between the

peptide solution and the air oscillate too much to determine this concentration (Fig. 4B). This variation could refer that the FAM-NFL aggregates and then disintegrates, which may explain its inability to form nanofibers.

When BIOT-NFL was dialyzed in water, peptide nanofiber structures were disassembled into peptide monomers or small polymers that passed through the dialysis membrane like individual peptide. The increasing amount of BIOT-NFL detected outside the dialysis membrane (UPLC measurements in Fig. 5C) in addition to the obvious decreasing of nanofibers length and density inside the dialysis tube at the end of dialysis (TEM observations in Fig. 5B) argue for such a disassociation of peptide nanofibers and their liberation during dialysis (many hours). As seen in Fig. S4, the diffusion of FAM- and nude-NFL-peptides (existing mainly as free peptide molecules with very few sort nanofilaments) was similar to the BIOT-NFL (Fig. 5C) indicating also that peptide molecules can disassociate from nanofibers and pass through dialysis membrane as a free peptide or small polypeptides. The similar diffusion profile of the different studied peptides could be explained by the membrane pore size used for dialysis (dialysis membrane cut-off = 6–8 kDa versus BIOT-NFL-peptide = 2.7 kDa) that has been chosen to ensure the free passage of peptide in its monomeric form (one or two molecules) and not in the form of nanofiber. Thus, even if the FAM- or nude-NFL exists mainly as a free peptide, their passage through the membrane takes place molecule by molecule, which may clarify their profile of diffusion. Another possible explanation could be that this NFL-peptide interacts with the dialysis tube, or a special compound from the dialysis tube, and thus affects its normal dialysis.

This experiment demonstrated a disassociation of BIOT-NFL-nanofibers due to the decrease of peptide concentration during dialysis, suggesting that the association between peptides molecules to form a long nanofiber is probably created by weak chemical bonds such as hydrogen-bonding which can be destructed (at this concentration of 1 mmol/L) due to dilution. Thus, the concentration seems to be significant to form peptide nanofibers as it could facilitate the side-by-side interaction of peptide monomers (Waku et al., 2018), while decreased concentrations disassemble the organized arrangement (Yan et al., 2008).

Using cell-penetrating peptides to facilitate the delivery of various biomolecules into the cell has been a topic of interest (Avci et al., 2018). Based on the target effect of this peptide against GBM cells and its capacity to form nanofibers, we aimed to conjugate the BIOT-NFL with other cargos to improve their uptake by cells. In this regard, we investigated the interaction between BIOT-NFL-nanofibers and gold nanoparticles. Gold nanoparticles coupled with streptavidin were used in this study to show the contribution of the BIOT-NFL-peptide coupled to gold particles to enter cells. The use of hyperthermia for the treatment of brain tumors was first reported in 1990 and it is under investigation for malignant glioma (Glaser et al., 2017). Different examples of the application of polymeric nanofibers for the delivery of metallic nanoparticles to tumor tissue, to produce heating and kill cancer cells, have been reported in the literature (Abid et al., 2019). The decoration of gold nanoparticles on the surface of peptide-nanofibers may also control particles aggregation and provide them additional stability (Maity et al., 2014; Abbas et al., 2022). The size of the gold nanoparticles in this study (6 nm) allows us to follow them by electron microscopy, but it is necessary to use other types of gold nanoparticles for photothermal treatment or to evaluate the plasmonic properties of gold under these conditions. From a therapeutic perspective, more quantitative and in-depth studies would be necessary to test such promising nano-objects *in vitro* and *in vivo* for their potential contribution to the anti-glioblastoma arsenal.

Interestingly, transmission electronic microscopic micrographs between gold nanoparticles and the BIOT-NFL-nanofibers showed an important interaction (Fig. 6A). When this system (gold nanoparticle / BIOT-NFL-nanofibers) was incubated with GBM cells, we observed an increase of the internalization of gold nanoparticles within the cells (Fig. 6C) compared to gold nanoparticles alone (Fig. 6B). Because of the

heaviness and the slowness of this technique, we were not able to carry out a thorough statistical study, but systematically we observed that the BIOT-NFL allows a better internalization of gold nanoparticles into F98 cells. In a parallel work, we also demonstrated an interaction between BIOT-NFL-nanofibers and another type of nanoparticles, lipid nanocapsules, with an enhancement of their internalization in GBM cells when conjugated to the peptide compared to nanocapsules alone (Grievau et al., 2022, submitted).

Altogether, the ability of BIOT-NFL to form nanofibers that can interact with other nanostructures, in addition to its capacity to directly target GBM cells, make this peptide a significant potential for the delivery of various cargoes into cells with limited toxicity.

5. Conclusion

Our data demonstrate the capacity of BIOT-NFL-peptide to form self-assembled peptide-nanofibers with approximately a diameter of 5 nm in wide and several micrometers in length. BIOT-NFL-nanofibers were detected in sterile water as well as other solutions (at physiological pH) that can mimic the cell environment or be used for peptide administration. These nanofibers were stable for long period of time (up to 30 days) in all studied solutions. A dense network of nanofibers was observed from BIOT-NFL-peptide when prepared at 1 mmol/L. However, peptide-nanofibers obtained from BIOT-NFL-peptide at this concentration were disassociated due to dilution effect, indicating that BIOT-NFL properties allowing peptide self-assembly, and disassembly. These results suggest that BIOT-NFL-peptide exhibits a concentration-dependent self-assembly behavior. The critical aggregation concentration (CAC) of the BIOT-NFL-peptide was determined at 10^{-4} mol/L, at this concentration the peptide starts to aggregate, then these aggregates can associate with each other to form self-assembled structure. Additionally, the β -sheet structure of NFL-peptide may facilitate the intermolecular interactions resulting in peptide self-assembly. However, further studies will be needed to better understand the mechanism of the self-assembly of this peptide. Moreover, our study proved an important interaction between BIOT-NFL-nanofibers and gold nanoparticles, with an enhancement of particles uptake by GBM cells when they are treated with this system (gold nanoparticle / BIOT-NFL-nanofibers), compared to gold nanoparticles alone.

At this point, we explored how naturally occurring nanofiber structures have inspired the development of novel delivery system owing to the interactions between peptide nanofibers and other nano cargos. Taken together, the BIOT-NFL-peptide could represent a promising therapeutic agent to target glioblastoma.

Funding

This work has been supported by Plan Cancer INSERM, Ligue contre le Cancer 49, and the PAUSE program from Collège de France.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgements

The authors acknowledge Florence Manero from SCIAM (Service Commun d'Imagerie et d'Analyses Microscopiques) for her help concerning the electron microscopy, Mélissa Giroudoux for her help for drop tensiometer experiment, and Jérôme Béjaud, Guillaume Bastiat and Patrick Saulnier for their helpful discussions and advice. Franck Artzner acknowledges Rennes Métropole for financial support of the X-ray setup.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijph.2022.100128>.

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