TITLE
Enhanced gene expression in the brain following intravenous administration of lactoferrin-bearing polypropylenimine dendriplex

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ABSTRACT
The possibility of using gene therapy for the treatment of brain diseases such as brain cancer, Alzheimer's and Parkinson's diseases, is currently hampered by the lack of gene delivery systems able to cross the blood-brain barrier and deliver DNA to the brain following intravenous administration.
On the basis that lactoferrin can effectively reach the brain by using specific receptors for crossing the blood-brain barrier, we propose to investigate if a lactoferrin-bearing generation 3- diaminobutyric polypropylenimine (DAB) dendrimer would allow the transport of plasmid DNA to the brain after intravenous administration.
In this work, we demonstrated that the conjugation of lactoferrin to the dendrimer led to an enhanced DNA uptake by 2.1-fold in bEnd.3 murine brain capillary endothelial cells compared to the unmodified dendriplex *in vitro*. *In vivo*, the intravenous administration of lactoferrin-bearing DAB dendriplex resulted in a significantly increased gene expression in the brain, by more than 6.4-fold compared to that of DAB dendriplex, while decreasing gene expression in the lung and the kidneys. Gene expression in the brain was significantly higher than in any other major organs of the body.
Lactoferrin-bearing generation 3 polypropylenimine dendrimer is therefore a highly promising delivery system for systemic gene delivery to the brain.

KEYWORDS
Brain delivery; blood-brain barrier; gene expression; dendrimer; lactoferrin

1. Introduction

Brain diseases, including glioma, Alzheimer's and Parkinson's diseases, currently represent one of the largest and fastest growing area of unmet clinical need. Brain cancer is the leading cause of cancer death in young people and accounts for more than one third of cancer deaths in children aged under 10. Alzheimer's disease is the most common type of dementia, affecting almost 500 000 people in the UK. The symptoms of this disease develop gradually and become more severe over the course of several years. In addition, there is currently no cure for Parkinson's disease, which affects 127 000 people in the UK [1-4].

Gene therapy has emerged as a promising therapeutic strategy, as the genetic basis for many of these diseases is known. However, the possibility of using genes as medicines to treat brain pathologies is limited by the lack of safe and efficacious delivery systems able to cross the blood-brain barrier (BBB) and to deliver DNA to the brain after intravenous administration, without secondary effects to healthy tissues. In addition, locally administered treatments fail to achieve a widespread gene expression in the target cells throughout the entire brain, which is necessary for a successful treatment of most brain pathologies [2-3, 5]. There is therefore an urgent need to develop safe and efficacious non-viral gene-based nanomedicines able to cross the BBB after intravenous administration, in order to ultimately provide better treatment options for brain diseases than currently available.

Despite acting as an entrance gateway to the brain, the BBB does possess specific receptor-mediated transport mechanisms that can potentially be exploited as a means to target drugs and genes to the brain. As iron can reach the brain by using transferrin (Tf) receptors overexpressed on the blood-brain barrier [6], targeting the transferrin receptor (TfR) strategy has been widely investigated for the delivery of drugs and genes to the brain [7-8]. Among the non-viral gene delivery systems currently under development, generation 3 diaminobutyric polypropylenimine dendrimer (DAB) appears to be particularly promising. We recently demonstrated that the intravenous administration of this dendrimer combined with a β-galactosidase expression plasmid led to gene expression in the brain, while decreasing the gene expression in the lung in a mouse model [9].

In order to further enhance the brain delivery efficacy of this dendrimer, we hypothesized that grafting lactoferrin instead of transferrin to DAB dendrimer could improve the delivery of DNA to the brain following intravenous administration. Lactoferrin (Lf) is a single chain cationic iron-binding protein belonging to the Tf family. It plays a role in the host defense against inflammation and infection and accumulates in the brain in patients suffering from neurodegenerative disorders [10-12]. It is capable of crossing the BBB by receptor-mediated transcytosis via the brain-specific Lf receptors, which belong to low-density lipoprotein receptor-related proteins (LRP) and are highly expressed on the BBB [13]. Previous studies have demonstrated that the gene expression of Lf-bearing polyamidoamide (PAMAM) dendriplex administered intravenously to mice was higher compared to that of PAMAM dendriplex [14]. Besides, Lf was shown to exhibit a higher BBB uptake compared to Tf, due to limited competition with endogenous ligands [15].

The objectives of this study were therefore to investigate if Lf-bearing DAB dendrimer could improve the delivery of DNA to the brain, *in vitro* and *in vivo* following intravenous administration.

2. Materials and methods
2.1. Cell lines and reagents

Generation 3- diaminobutyric polypropylenimine dendrimer (PPI G3; DAB), lactoferrin (Lf), dimethylsuberimidate and all other chemicals that are not specifically mentioned below were purchased from Sigma Aldrich (Poole, UK). DAB conjugated to Lf (DAB-Lf) was synthesized and characterized as described in our previous study [16], using a similar technique as used for DAB-Tf [17]. The expression plasmids encoding β-galactosidase

(pCMVsport β-galactosidase) and tdTomato (pCMV-tdTomato) were respectively obtained from Invitrogen (Paisley, UK) and Clontech (Mountain View, CA). The expression plasmid encoding luciferase (pEF1α-Luc) was constructed by subcloning the elongation factor 1 alpha (EF1α) promoter from pEF1/Myc-His vector (Invitrogen, Paisley, UK) into the promoterless pGL3-Basic vector (Promega, Southampton, UK). All these plasmids were propagated in *E. coli* and purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany). Vectashield® mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) and passive lysis buffer were respectively purchased from Vector Laboratories (Peterborough, UK) and Promega (Southampton, UK). Tissue-Tek® optimal cutting temperature (OCT) compound came from VWR (Lutterworth, UK). Label IT® fluorescein- Nucleic Acid Labeling kits were obtained from Cambridge Biosciences (Cambridge, UK). Firefly D-luciferin was purchased from Caliper Life Sciences (Hopkinton, MA). bEnd.3 murine brain capillary endothelial cell line came from LGC Standards (Teddington, UK), while cell culture media were obtained from Invitrogen (Paisley, UK).

2.2. In vitro biological characterization
2.2.1. Cell culture
Immortalized bEnd.3 cells overexpressing Lf receptors were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 0.5% (v/v) penicillin-streptomycin. Cells were cultured in a humid atmosphere of 5% carbon dioxide, at 37°C.

2.2.2. Cellular uptake
Imaging of the cellular uptake of the DNA carried by DAB-Lf was carried out by epifluorescence microscopy. Labeling of plasmid DNA with the fluorescent probe fluorescein was performed using a Label IT® Fluorescein Nucleic Acid Labeling kit, as described by the manufacturer. bEnd.3 cells were seeded on coverslips in 6-well plates (10^4 cells/ well) and grown at 37°C for 72 h. The cells were then incubated for different durations (1h, 2h, 3h, 4h) with fluorescein-labeled DNA (2.5 μg/well) complexed to DAB-Lf (dendrimer: DNA weight ratio of 2:1). The slides were then washed three times with 3 mL PBS and fixed with 2 mL methanol for 10 min. Upon staining of the nuclei with DAPI, the cells were examined using an E600FN Upright Epifluorescence microscope (Nikon, Tokyo, Japan). DAPI was excited with the 365 nm CoolLED pE excitation system (bandwidth: 435-485 nm), whereas fluorescein was excited with the 470 nm CoolLED pE excitation system (emission bandwidth: 515-555 nm).

Once the treatment duration allowing maximal DNA uptake was determined, a similar procedure was performed to compare the cellular uptake of fluorescein-labeled DNA (2.5 μg/well) complexed to DAB-Lf and DAB (dendrimer: DNA weight ratios respectively of 2:1 and 5:1 [16, 18]) during the optimized treatment duration. Control samples were treated with naked DNA or remained untreated.

Quantification of cellular uptake was also carried out by flow cytometry. bEnd3 cells were seeded in 6-well plates (1.6 x 10^5 cells/well) and grown at 37 °C for 72 h. The cells were then treated with fluorescein-labeled DNA (5 μg DNA/well), alone or complexed to DAB-Lf and DAB (dendrimer: DNA weight ratios respectively of 2:1 and 5:1). Untreated cells served as a negative control. After 2 h incubation with the treatments, single cell suspensions were prepared, washed (2 mL PBS pH 7.4 per well) and pelleted (378 g for 8 min) 3 times, before being analyzed using a FACSCanto® flow cytometer (BD, Franklin Lakes, NJ). Ten thousand cells (gated events) were counted for each sample. Their mean fluorescence intensity was analyzed with FACSDiva® software (BD, Franklin Lakes, NJ).

2.2.3. Mechanisms of cellular uptake of DNA complexed to DAB-Lf dendriplex
The mechanisms involved in the cellular uptake of DNA complexed to DAB-Tf dendriplex were investigated by pre-treatment with uptake inhibitors and escalating concentrations of endogenous ligands (free Lf). Cells were seeded and grown as described above. After

removal of the medium, they were pre-treated with phenylarsine oxide (10 µmol/L), filipin (5 µg/mL), colchicine (10 µmol/L), poly-L-lysine (400 µg/mL) and free Lf at concentrations ranging from 2.5 to 20 µmol/L for 10 min at 37°C. The cells were then treated with fluorescein-labeled DNA (respectively 2.5 and 5 µg/well for qualitative and quantitative analysis) complexed to DAB-Lf for a duration of 2h, before being washed and processed for fluorescence microscopy and flow cytometer analysis as described above.

2.2.4. In vitro transfection

Transfection efficacy of the DNA carried by DAB-Lf dendrimer was assessed with a plasmid DNA encoding β-galactosidase (pCMV βgal), using a β-galactosidase transfection assay. bEnd.3 cells were seeded at a density of 2 000 cells/well in 96-well plates (n=15). After 72h incubation, the cells were treated with DAB-Lf complexed to plasmid DNA encoding β-galactosidase, at the dendrimer: DNA weight ratio of 2:1, which we previously showed to give the highest transfection on other cancer cell lines [16]. Naked DNA served as a negative control, DAB-DNA (dendrimer: DNA weight ratio 5:1) [18] served as a positive control. DNA concentration (10 µg/mL) was kept constant for all the formulations tested. After 72h incubation, cells were lysed with 1X passive lysis buffer (PLB) (50 µL/well) for 20 min. The cell lysates were subsequently analyzed for β-galactosidase expression. Briefly, 50 µL of the assay buffer (2 mM magnesium chloride, 100 mM mercaptoethanol, 1.33 mg/mL ONPG, 200 mM sodium phosphate buffer, pH 7.3) was added to each well containing the lysate. After 2 h incubation at 37°C, the absorbance of the samples was read at 405 nm with a Multiscan Ascent® plate reader (Thermo Scientific, Waltham, MA).

2.3. In vivo study
2.3.1. Animals

Female BALB/c mice were housed in groups of five at 19°C to 23°C with a 12-h light-dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded, B&K Universal, Grimston, United Kingdom) with mains water *ad libitum*. Experimental work described below was carried out in accordance with UK Home Office regulations and approved by the local ethics committee.

2.3.2. Biodistribution of gene expression

The biodistribution of gene expression was also visualized by bioluminescence imaging, using an IVIS Spectrum® (PerkinElmer, Waltham, MA).
To determinate the treatment duration leading to the highest gene expression in the brain, female BALB/c mice (n= 3, initial mean weight: 20 g) were intravenously injected with a single dose of DAB-Lf carrying luciferase-expression plasmid (50 µg of DNA). They were then intraperitoneally injected with the luciferase substrate D-luciferin (150 mg/kg body weight) after various treatment durations and anaesthetized by isoflurane inhalation. Light emission was measured 10 min after injection of the D-luciferin solution, for 2 min, using Living Image® software (PerkinElmer, Waltham, MA). The resulting pseudo-color images represent the spatial distribution of photon counts within the animal. Identical illumination settings were used for acquiring all images [19].
A similar procedure was then performed at the treatment duration leading to the highest gene expression in the brain, to compare the distribution of gene expression resulting from the single intravenous injection of DAB-Lf and DAB dendriplexes encoding luciferase (50 µg of DNA).
Biodistribution of gene expression was also quantified using a β-galactosidase reporter gene expression assay. Groups of mice (n= 5, initial mean weight: 20 g) were injected intravenously with a single dose of DAB-Lf and DAB dendriplexes encoding β-galactosidase (50 µg of DNA). They were sacrificed at the treatment duration leading to the highest gene expression in the brain. Their organs were removed, immediately frozen

in liquid nitrogen, before being analyzed for their β-galactosidase levels as previously described [20].

2.3.3. Distribution of gene expression within the brain
Distribution of gene expression within the brain was qualitatively assessed by fluorescence microscopy imaging of the brain sections of mice treated with DAB-Lf dendriplex encoding tdTomato. Mice were intravenously injected with a single dose of DNA encoding tdTomato, naked or complexed to DAB-Lf and DAB dendrimers (50 µg of DNA). They were sacrificed 24 h after injection and their brains were removed, immediately frozen on dry ice, before being embedded in Tissue-Tek® optimal cutting temperature (OCT) compound. Coronal sections were cut on a cryostat (Thermo Scientific, Hemel Hempstead, UK) at a thickness of 15 µm in different brain areas (anterior, median and posterior), fixed in ice-cold acetone for 5-10 min, in -20ºC methanol for 5-10 min. They were then washed and permeabilized in PBS/0.1% Tween-20 for 10 min before being mounted with Vectashield® medium containing DAPI. The brain sections were then examined using an E600FN Upright Epifluorescence microscope. Positivity for tdTomato expression in the brain was assessed at excitation wavelengths of 530-635 nm and emission wavelengths of 605-655 nm.

2.4. Statistical Analysis
Results were expressed as means ± standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison post-test (Minitab® software, State College, PE). Differences were considered statistically significant for P values lower than 0.05.
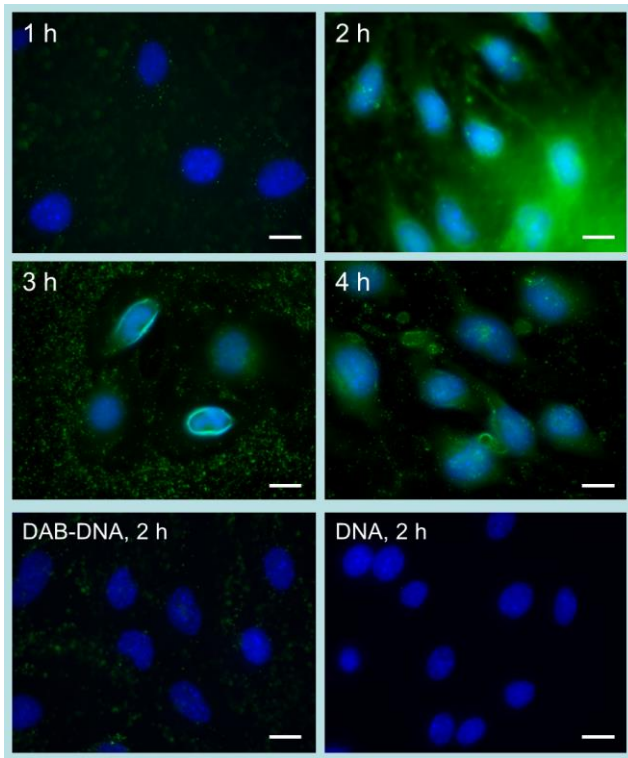
3. Results and discussion
3.1. In vitro biological characterization
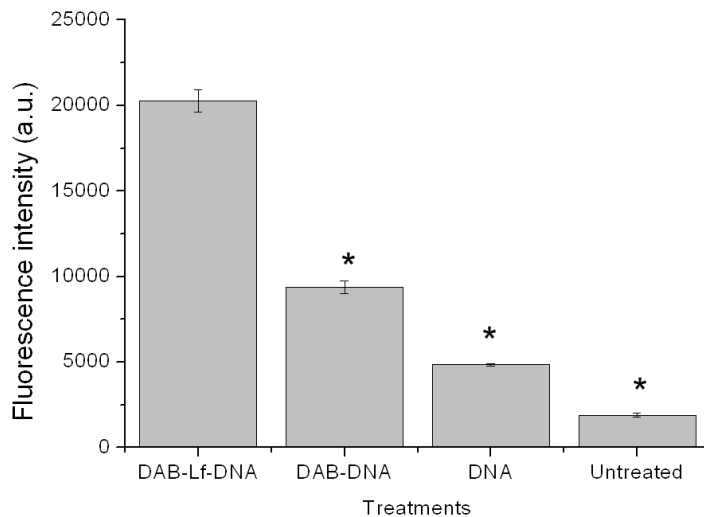### 3.1.1. Cellular uptake
The uptake of fluorescein-labeled DNA complexed to DAB-Lf by bEnd.3 cells was qualitatively confirmed by epifluorescence microscopy. DNA uptake in bEnd.3 cells appeared to be most pronounced after incubation of the cells with DAB-Lf dendriplex for 2 h (Fig. 1). Fluorescein-labeled DNA was still disseminated in the cytoplasm of the cells 4 h after the start of the treatment.

We then evaluated the cellular uptake of fluorescein-labeled DNA either complexed to DAB-Lf and DAB, or as a solution, following 2 h incubation (Fig. 1). Cells treated with DAB dendriplex or DNA solution did not show any fluorescein-derived fluorescence, highlighting the need of a targeted delivery system to carry DNA to bEnd.3 cells.

These cellular uptake results were quantitatively confirmed by flow cytometry (Fig. 2, Supplementary Fig. 1). Treatment with DAB-Lf dendriplex (20 249 ± 649 arbitrary units (a.u.)) resulted in the highest cellular fluorescence, which was respectively 2.1-fold and 4.1-fold higher than that observed after treatment with DAB dendriplex (9 368 ± 383 a.u.) and DNA solution (4 839 ± 59 a.u.). For example, in one of the repeats, both the percentage of fluorescein–positive cells as well as the mean fluorescence intensity increased from 45% to 54% (with corresponding mean fluorescence intensities of 16 301 and 36 067 a.u.) after conjugation with Lf (Supplementary Fig. 1).

Figure 1. Epifluorescence microscopy imaging of the cellular uptake of fluorescein-labeled DNA (2.5 µg/ well) complexed with DAB-Lf after incubation for 1h, 2h, 3h or 4h with bEnd.3 cells, or complexed with DAB or in solution after incubation for 2h with the cells (Blue: nuclei stained with DAPI (excitation: 365 nm, emission bandwidth: 435-485 nm), green: fluorescein-labeled DNA (excitation: 470 nm, emission bandwidth: 515-555 nm) (Bar: 10 µm).

Figure 2. Flow cytometry quantification of the cellular uptake of fluorescein- labeled DNA (5 µg/ well) either complexed with DAB-Lf, DAB or in solution, after incubation for 2 hours with bEnd.3 cells (n=5). * : $P < 0.05$ compared with DAB-Lf-DNA.
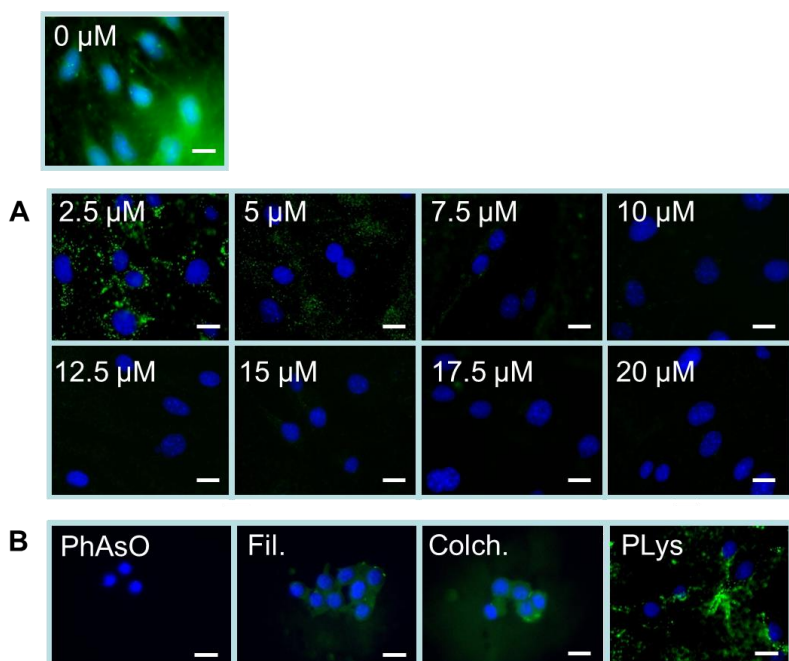
In this study, we demonstrated that the grafting of Lf on the dendrimer DAB enhanced DNA uptake by bEnd.3 murine brain capillary endothelial cells, compared to control dendriplex and naked DNA treatments. These results were in accordance with previous data obtained by Hu and colleagues [21], who reported that treating bEnd.3 cells with Lf-bearing PEG-poly-(lactide) nanoparticles encapsulating coumarin-6 increased the cellular uptake of the drug by 1.45-fold compared to non-targeted nanoparticles. In another study,

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Lf- conjugated polymersomes led to similar effects, as their cellular uptake by bEnd.3 cells was 1.56-fold higher than that of the non-conjugated polymersomes [22].

When compared with our previous experiments with DAB-Tf, the conjugation of Lf, instead of Tf, to DAB dendrimer resulted in a slight increase of DNA uptake by bEnd.3 cells, from 1.4-fold to 2.1-fold [9]. This outcome was in line with the results previously described by Huang and colleagues [14] when comparing the cellular uptakes of Lf-bearing and Tf-bearing PAMAM-PEG dendrimers by brain capillary endothelial cells.

3.1.2. Mechanisms of cellular uptake of DNA complexed to DAB-Lf dendriplex

Pre-treatment of the bEnd.3 cells with various concentrations of free Lf significantly decreased the cellular uptake of fluorescein-labeled DNA complexed to DAB-Lf at concentrations as low as 2.5 µM and remained at similar levels up to 17.5 µM (Fig. 3 and 4). At an Lf concentration of 20 µM, the cellular uptake of fluorescently-labeled DNA was 4.3-fold lower than that observed with DAB-Lf dendriplex without pre-Lf treatment (respectively 4 665 ± 96 a. u. and 20 249 ± 649 a. u.) (Fig. 4).

Figure 3. Epifluorescence microscopy imaging of the bEnd.3 cellular uptake of fluorescein-labeled DNA (2.5 µg/ well) complexed with DAB-Lf, following pre-treatment with various concentrations of free Lf (ranging from 2.5 µM to 20 µM) (A) and following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide ("PhAsO"), filipin ("Fil."), colchicine ("Colch.") and poly-L-lysine ("PLys") (B). (Blue: nuclei stained with DAPI (excitation: 365 nm, emission bandwidth: 435-485 nm), green: fluorescein-labeled DNA (excitation: 470 nm, emission bandwidth: 515-555 nm) (Bar: 10 µm).

The cellular uptake of fluorescein-labeled DNA complexed to DAB-Lf was partially inhibited by phenylarsine oxide, filipin, colchicine and poly-L-lysine (Fig. 3 and 4). Phenylarsine oxide caused the most significant inhibition, with a cellular uptake respectively decreased by 8.2-fold compared to that observed with DAB-Lf dendriplex without inhibitory treatment (2 450 ± 71.56 a.u. following pre-treatment with phenylarsine oxide). Filipin and Colchicine appear to be partial inhibitors, leading to a cellular uptake decrease by respectively 2-fold and 2.1-fold compared to DAB-Lf dendriplex without pre-treatment (respectively 9 773 ± 199 a. u. and 9 579 ± 89 a.u. following pre-treatment with filipin and colchicine). Poly-L-Lysine caused the least inhibition with 1.2-fold decrease in the cellular uptake (15 764 ± 690 a.u following pre-treatment with poly-L-Lysine).
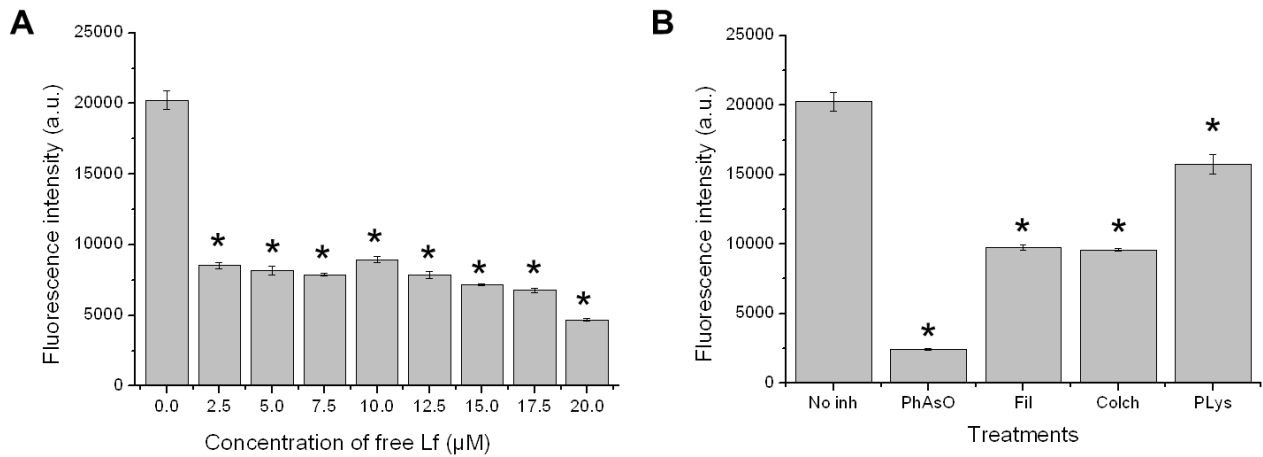
Figure 4. Flow cytometry quantification of the bEnd.3 cellular uptake of fluorescein-labeled DNA (5 µg/ well) complexed with DAB-Lf, following pre-treatment with various concentrations of free Lf (ranging from 2.5 µM to 20 µM) (A) and following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide ("PhAsO"), filipin ("Fil."), colchicine ("Colch.") and poly-L-lysine ("PLys") (B) (n=5), * : P <0.05 compared with DAB-Lf-DNA.

Each of these inhibitors acts on a different endocytic mechanism on the BBB. Phenylarsine oxide has been reported to be an inhibitor of clathrin-mediated endocytosis [23]. Filipin blocks the caveolae-mediated process in non-specific adsorptive endocytosis [24]. Colchicine is known to inhibit macropinocytosis [25], which provides non-specific endocytosis of macromolecules, whereas cationic poly-L-Lysine acts as an uptake inhibitor for cationic delivery systems. The cellular uptake of DNA complexed to DAB-Lf was therefore mainly related to clathrin-mediated endocytosis (which is a requisite for lactoferrin receptor-mediated endocytosis) and to a lesser extent caveolae-mediated endocytosis and macropinocytosis.

Pre-treatment of cells with escalating amounts of free Lf led to competition between DAB-Lf dendriplex and free Lf for binding to Lf receptors, suggesting that the internalization of the DNA complexed to DAB-Lf is principally due to Lf receptors- mediated endocytosis. These results are in accordance with a study by Huang and colleagues [14] in which they reported a significant inhibition of the cellular uptake of Lf-bearing PAMAM-PEG dendrimer when BCECs were pre-treated with phenylarsine oxide, fillipin, colchicine and excess free Lf.

3.1.3. In vitro transfection

The treatment of bEnd.3 cells with DAB-Lf dendriplex led to a 2.1-fold increase of gene expression compared to DAB dendriplex ($6.01 \times 10^{-3} \pm 0.42 \times 10^{-3}$ U/mL and $2.85 \times 10^{-3} \pm 0.21 \times 10^{-3}$ U/mL respectively for DAB-Lf and DAB dendriplexes) and 7-fold higher than that observed following treatment with naked DNA ($0.85 \times 10^{-3} \pm 0.01 \times 10^{-3}$ U/mL) (Fig. 5). The cells treated with naked DNA did not show any significant increase in gene expression compared to untreated cells.

The enhanced β-galactosidase expression following treatment of the cells with DAB-Lf dendriplex most likely resulted from the increased cellular uptake obtained after this treatment. Both increases were of the same magnitude (2.1-fold for both cellular uptake and gene expression compared to non-targeted DAB dendriplex treatment). Although the differences between treatments were less pronounced in our study, our results were in accordance with previous data obtained by Huang and colleagues [14], who revealed that luciferase gene expression in the brain capillary endothelial cells was 3.6-fold higher after treatment with Lf-bearing PEG-PAMAM dendriplex compared to that observed with the

non-targeted dendriplex. When compared with our previous experiments with DAB-Tf, the conjugation of Lf on DAB dendriplex led to a slight increase of gene expression, from1.3-fold to 2.1-fold, compared to that observed following treatment with Tf-bearing DAB dendriplex [9].
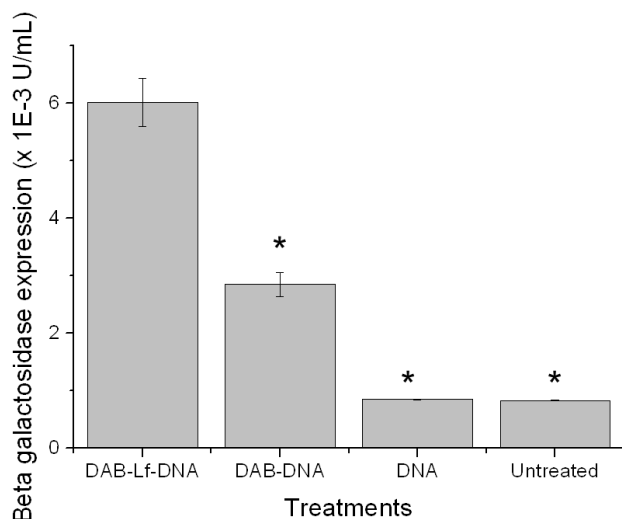
Figure 5. Transfection efficacy of DAB-Lf and DAB dendriplexes in bEnd.3 cells. DAB-Lf and DAB dendriplexes were dosed at their optimal dendrimer: DNA ratio of 2:1 and 5:1 respectively. Results are expressed as the mean ± SEM of three replicates (n=15). * : P <0.05 compared with DAB-Lf-DNA.
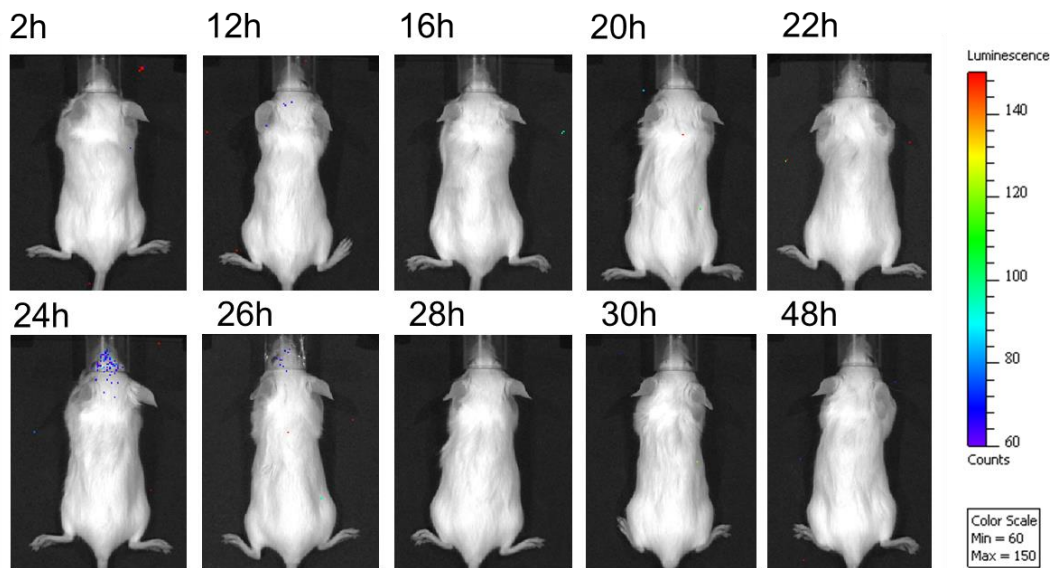
3.2. In vivo study

Distribution of gene expression following intravenous injection of DAB-Lf dendriplex encoding luciferase was first qualitatively assessed by luminescence imaging, at various treatment durations. Gene expression appeared to be mainly located in the brain of the mice. The highest gene expression level was found 24 h following injection of DAB-Lf dendriplex (Fig. 6).
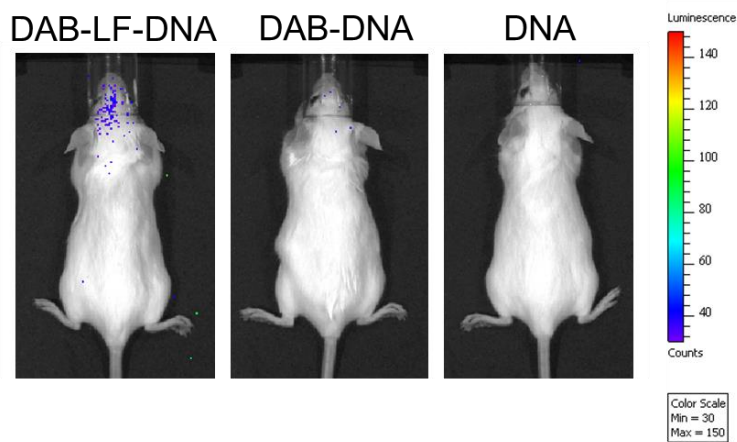
Gene expression following administration of DAB-Lf dendriplex was then compared to that observed following administration of DAB dendriplex and DNA only, 24h after administration of the treatments. The level of gene expression in the brain appeared to be highest following treatment with DAB-Lf dendriplex (Fig. 7). In addition, luciferase expression appeared to be very limited in organs other than brain, as well as in the brain before 22 h and after 26 h. This might be explained by the threshold of the technique that probably allowed only the most intensely luminescent tissues to be analyzed.

These results were confirmed by quantification of gene expression in the major organs of the mice. The intravenous administration of DAB-Lf dendriplex led to gene expression mainly in the brain (116.1 ± 9.0 mU and 17.9 ± 11.3 mU β-galactosidase per organ), followed by the liver (70.9 ± 14.9 mU β-galactosidase per organ), the spleen (17.6 ± 15.0 mU β-galactosidase per organ) and then the heart (4.2 ± 4.2 mU β-galactosidase per organ) (Fig. 8). The conjugation of Lf to DAB significantly increased by more than 6.4-fold the gene expression in the brain compared to that of DAB dendriplex (17.9 ± 11.3 mU β-galactosidase per organ). It also decreased the gene expression in the lung and the kidneys, which were respectively 46.30 ± 10.6 mU β-galactosidase per organ and 28.9 ± 18.7 mU β-galactosidase per organ in the lung and the kidneys for DAB dendriplex. However, there was no significant differences between the levels of β-galactosidase gene expression in liver and spleen following treatment with DAB-Lf dendriplex, DAB-dendriplex and DNA solution (45.1 ± 9.0 mU and 45.1 ± 6.9 β-galactosidase per organ in the liver for respectively DAB dendriplex and DNA solution, 17.6 ± 15.0 mU, 33.8 ± 24.3 mU and 10.5
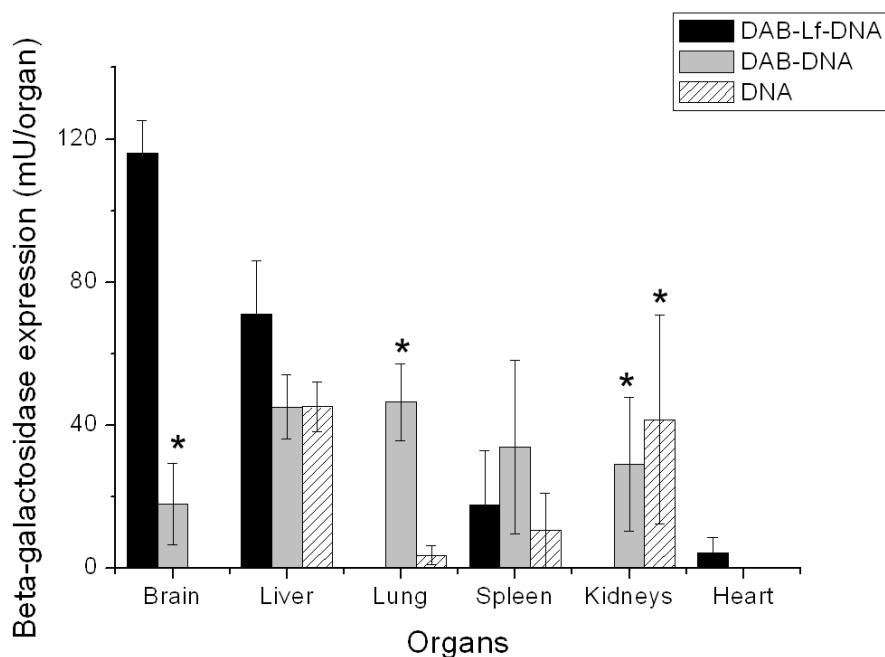
± 10.5 mU β-galactosidase in the spleen for respectively DAB-Lf dendriplex, DAB dendriplex and DNA solution).

Figure 6. Bioluminescence imaging of gene expression after intravenous administration of DAB-Lf dendriplex (50 µg DNA administered). The mice were imaged using the IVIS Spectrum at various durations after injection of the treatment. The scale indicates surface radiance (photons/s/cm^2/steradian).

Figure 7. Bioluminescence imaging of gene expression after intravenous administration of DAB-Lf and DAB dendriplexes (50 µg DNA administered). (Controls: DNA solution). The mice were imaged using the IVIS Spectrum 24h after injection of the treatments. The scale indicates surface radiance (photons/s/cm^2/steradian).
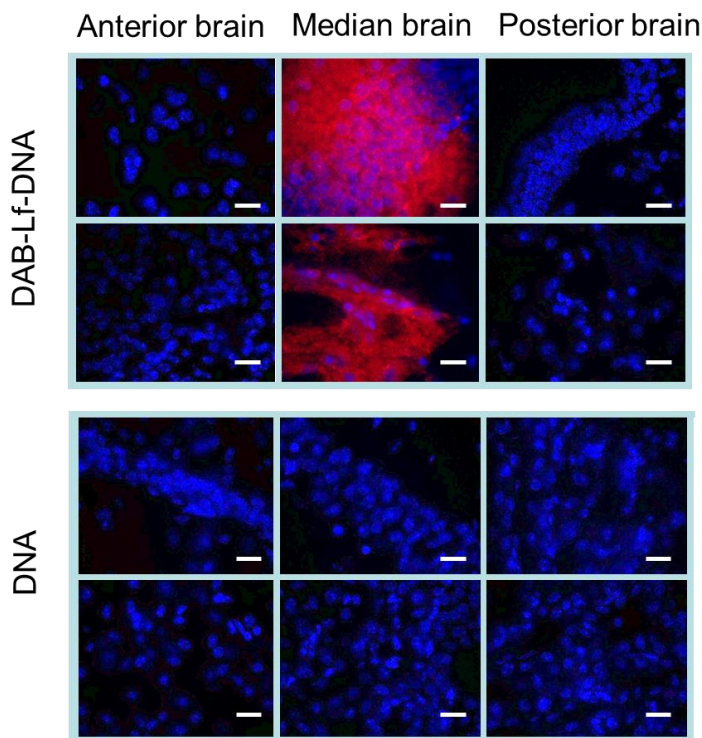
Figure 8. Biodistribution of gene expression after a single intravenous administration of DAB-Lf and DAB dendriplexes (50 µg DNA administered). Results were expressed as milliunits β-galactosidase per organ (n=5). * : P <0.05 compared with DAB-Lf-DNA for each organ.

In a separate experiment using DAB-Lf dendriplex encoding tdTomato instead of β-galactosidase, tdTomato gene expression following administration of DAB-Lf dendriplex was visible within the brain in the dentate gyrus and in the granule cell layer of the hippocampus, in the median brain section (Fig. 9). It appeared to be distributed in the cytoplasm of the cells rather than in their nuclei. By contrast, there was no gene expression visible in the median brain following injection of naked DNA, or in the anterior and posterior sections of the brain following any of these two treatments.

This communication presents evidence that the intravenous injection of DAB-Lf dendriplex resulted in an enhanced gene expression in the brain, which was significantly higher than in any other major organs of the body. It also decreased gene expression in the lung and the kidneys, compared to that observed following treatment with DAB dendriplex.
Another group has already demonstrated gene transfer capabilities of Lf-bearing gene delivery systems following intravenous administration, but with much smaller gene expression in the brain and much larger non-specific expression in other organs. The intravenous administration of Lf-bearing PAMAM-PEG dendriplex to mice resulted in a 5.2-fold increase of the luciferase gene expression in the brain compared to the non-targeted dendriplex [14]. However, high levels of luciferase gene expression were also observed in the heart, spleen, lung and kidneys. Gene expression was the highest in the heart among the various tested organs.

Figure 9. Epifluorescence microscopy imaging of the distribution of gene expression within the anterior, median and posterior brain areas after a single intravenous injection of tdTomato- encoded DNA (50 µg) either complexed with DAB-Lf or in solution (Blue: nuclei stained with DAPI (excitation: 365 nm, emission bandwidth: 435-485 nm), red: tdTomato expression (excitation bandwidth: 530-635 nm, emission bandwidth: 605-655 nm) (Bar: 10 µm).

Other studies using Lf-bearing drug delivery systems have also reported an increase of the drug delivery to the brain after intravenous injection, however associated with an increase of the amount of drug delivered to non-specific organs. For example, the administration of Lf-bearing PEG-liposomes encapsulating coumarin-6 resulted in a 1.4-fold increase in the brain uptake of the drug compared to that observed with PEG-liposomes, but non-specific uptake in spleen also increased [26]. In another study, Lf -bearing β-cyclodextrin nanocarrier increased the brain uptake of the delivery system by 3.5-fold compared to the non-targeted carrier [27]. However, its highest uptake was found to be in the liver, spleen and kidneys. These variations in biodistribution when using different delivery systems might be the consequence of different delivery system-dependent contributions of receptor- and adsorptive-mediated mechanisms to the brain uptake of Lf-bearing vectors. However, this hypothesis needs to be further investigated.

Following administration of DAB-Lf dendriplex, tdTomato gene was expressed in the hippocampus, which was consistent with the previous study by Huang and colleagues using Lf-bearing PAMAM-PEG dendriplex [14]. The hippocampus plays an important role in consolidating information from short-term memory into long-term memory. It is a primary site for Alzheimer's pathology, which makes gene expression in this brain area particularly interesting for future therapeutic developments.

When compared with DAB-Tf, gene expression in the brain following administration of Lf-bearing DAB dendriplex was 3-fold higher [9]. Targeting Lf receptors present advantages compared to Tf receptors regarding crossing the BBB. Firstly, it has been reported that the specific binding of Lf with Lf receptors on BBB was not affected by the low endogenous Lf under physiological conditions, which is not the case for Tf [28]. Furthermore, Lf-mediated penetration of BBB is unidirectional, which might increase accumulation of the

dendriplexes in the brain [29] and thus makes Lf a highly promising targeting moiety for brain delivery.

In conclusion, the grafting of lactoferrin to DAB dendriplex is shown here to enhance DNA uptake in bEnd.3 murine brain capillary endothelial cells compared to the unmodified dendriplex *in vitro*. *In vivo*, the intravenous injection of lactoferrin-bearing DAB dendriplex resulted in an enhanced gene expression in the brain, which was significantly higher than in any other major organs of the body. It also decreased gene expression in the lung and the kidneys, compared to that observed following treatment with DAB dendriplex. Lactoferrin-bearing DAB dendrimer is therefore a highly promising nanocarrier for gene delivery to the brain following intravenous administration.

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FIGURE CAPTIONS

Figure 1. Epifluorescence microscopy imaging of the cellular uptake of fluorescein-labeled DNA (2.5 µg/ well) complexed with DAB-Lf after incubation for 1h, 2h, 3h or 4h with bEnd.3 cells, or complexed with DAB or in solution after incubation for 2 h with the cells (Blue: nuclei stained with DAPI (excitation: 365 nm, emission bandwidth: 435-485 nm), green:

fluorescein-labeled DNA (excitation: 470 nm, emission bandwidth: 515-555 nm) (Bar: 10 μm).

Figure 2. Flow cytometry quantification of the cellular uptake of fluorescein- labeled DNA (5 μg/ well) either complexed with DAB-Lf, DAB or in solution, after incubation for 2 hours with bEnd.3 cells (n=5). * : $P < 0.05$ compared with DAB-Lf-DNA.

Figure 3. Epifluorescence microscopy imaging of the bEnd.3 cellular uptake of fluorescein-labeled DNA (2.5 μg/ well) complexed with DAB-Lf, following pre-treatment with various concentrations of free Lf (ranging from 2.5 μM to 20 μM) (A) and following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide ("PhAsO"), filipin ("Fil."), colchicine ("Colch.") and poly-L-lysine ("PLys") (B). (Blue: nuclei stained with DAPI (excitation: 365 nm, emission bandwidth: 435-485 nm), green: fluorescein-labeled DNA (excitation: 470 nm, emission bandwidth: 515-555 nm) (Bar: 10 μm).

Figure 4. Flow cytometry quantification of the bEnd.3 cellular uptake of fluorescein-labeled DNA (5 μg/ well) complexed with DAB-Lf, following pre-treatment with various concentrations of free Lf (ranging from 2.5 μM to 20 μM) (A) and following pre-treatment with various cellular uptake inhibitors: phenylarsine oxide ("PhAsO"), filipin ("Fil."), colchicine ("Colch.") and poly-L-lysine ("PLys") (B) (n=5), * : $P < 0.05$ compared with DAB-Lf-DNA.

Figure 5. Transfection efficacy of DAB-Lf and DAB dendriplexes in bEnd.3 cells. DAB-Lf and DAB dendriplexes were dosed at their optimal dendrimer: DNA ratio of 2:1 and 5:1 respectively. Results are expressed as the mean ± SEM of three replicates (n=15). * : $P < 0.05$ compared with DAB-Lf-DNA.

Figure 6. Bioluminescence imaging of gene expression after intravenous administration of DAB-Lf dendriplex (50 μg DNA administered). The mice were imaged using the IVIS Spectrum at various durations after injection of the treatment. The scale indicates surface radiance (photons/s/cm^2/steradian).

Figure 7. Bioluminescence imaging of gene expression after intravenous administration of DAB-Lf and DAB dendriplexes (50 μg DNA administered). (Controls: DNA solution). The mice were imaged using the IVIS Spectrum 24h after injection of the treatments. The scale indicates surface radiance (photons/s/cm^2/steradian).

Figure 8. Biodistribution of gene expression after a single intravenous administration of DAB-Lf and DAB dendriplexes (50 μg DNA administered). Results were expressed as milliunits β-galactosidase per organ (n=5). * : $P < 0.05$ compared with DAB-Lf-DNA for each organ.

Figure 9. Epifluorescence microscopy imaging of the distribution of gene expression within the anterior, median and posterior brain areas after a single intravenous injection of tdTomato- encoded DNA (50 μg) either complexed with DAB-Lf or in solution (Blue: nuclei stained with DAPI (excitation: 365 nm, emission bandwidth: 435-485 nm), red: tdTomato expression (excitation bandwidth: 530-635 nm, emission bandwidth: 605-655 nm) (Bar: 10 μm).