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RESEARCH PAPER

Fatty Acid-Based Strategy for Efficient Brain Targeted Gene Delivery

Jie Shen • Mei Yu • Qinggang Meng • Jin Li • Yifan Lv • Weiyue Lu

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ABSTRACT

Purpose To investigate a fatty acid-based strategy for efficient brain targeted gene delivery and to understand mechanism(s) of this small molecule-mediated brain gene delivery strategy.

Methods A series of fatty acids (FAs) were conjugated with polyethylenimine (PEI_{25k}). A near-infrared fluorescence probe, IR820, was used to study *in vivo* and *ex vivo* brain targeting ability of these fatty acid-PEI conjugates (FA-PEIs). Brain uptake of FA-PEI_{25k}/rhodamine-6-isothiocyanate (RITC)-labeled DNA nanoparticles was investigated *via* a fluorescence imaging method. Moreover, pEGFP was used as a model gene to study *in vitro* and *in vivo* transfection effect of the ideal FA-PEI_{25k} conjugate.

Results FA modification did not have interference with the complexation between DNA and the PEI_{25k}. The FA-PEI_{25k} conjugates showed excellent brain targeting ability compared with unmodified PEI_{25k}. Among these FA-PEI_{25k} conjugates studied, myristic acid (MC)-PEI_{25k} showed sustained brain distribution profile and higher brain DNA uptake. Furthermore, MC-PEI_{25k}/pEGFP nanoparticles was able to achieve efficient *in vitro* and *in vivo* gene transfection. GFP expression was observed at different brain regions *in vivo*.

Conclusions These results demonstrated that the small molecule fatty acid, particularly myristic acid-based brain gene delivery strategy, is promising to mediate efficient gene transfection in the brain.

KEY WORDS brain targeting · fatty acid · gene transfection · myristic acid · polyethylenimine

ABBREVIATIONS

BBB	Blood–brain barrier
CNS	Central nervous system
DAPI	4,6-diamidino-2-phenylindole
EACA	6-aminohexanoic acid
EGFP	Enhanced green fluorescent protein
FA	Fatty acid
GDNF	Glial cell line-derived neurotrophic factor
LC	Lauric acid
MC	Myristic acid
NIR	Near infrared
OC	Octanoic acid
PC	Palmitic acid
PEI	Polyethyleneimine
RITC	Rhodamine-6-isothiocyanate
SC	Stearic acid

INTRODUCTION

Variety of brain diseases such as Alzheimer's disease (AD), the neurodegeneration of Parkinson's disease (PD) as well as

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brain cancers have been greatly threatening human health. Many of these brain diseases do not respond very well to conventional small molecule therapeutics (1,2) and consequently, gene therapy offers an alternative to current pharmacologic and surgical treatments for these diseases. Although gene therapy has shown promising for the treatment of both genetic and acquired brain diseases (2–4), the presence of the blood–brain barrier (BBB) dramatically prevents brain uptake of gene therapeutics and thereby hampers brain gene therapy.

The BBB formed by a tight arrangement of brain capillary endothelial cells (BCECs), acts as a protective barrier for the central nervous system (CNS) due to its tight junction structure as well as the lack of fenestration (5,6). It prevents the brain uptake of more than 98% of all potential neurotherapeutics and has been a major challenge in the field of brain gene delivery (7,8). Although invasive strategies such as craniotomy and intracerebral injection can bypass the BBB and achieve brain gene expression, these strategies are considered to be highly invasive and unable to deliver exogenous genes to the global areas of the brain (9). Over the past decade, numerous efforts have been made to develop efficient, non-viral brain gene delivery strategies based on cationic lipids or polymers (4,10,11). Among all non-viral gene delivery vectors, polyethylenimine (PEI) is one of the most effective and commercially available polymeric gene delivery carriers (12). Highly positively charged PEI can complex and condense negatively charged DNA to protect DNA from degradation, as well as allow DNA to escape from endosomes *via* the "proton sponge" effect. Consequently, the PEI can achieve high gene transfection *in vitro* and *in vivo* (13). However, the PEI itself has been found to be inefficient to carry gene therapeutics to cross the BBB (4,12). Accordingly, brain-targeted ligands (such as rabies virus glycoprotein (RVG)) have been used to modify PEI to facilitate brain gene delivery (14).

Myristic acid (MC) is a saturated fatty acid with a 14-carbon moiety, which can anchor a peptide across biological membranes with no registered toxicity (15). Myristoylated polyarginine was able to cross the BBB and accumulate in neurons with occasional localization in astrocytes and endothelial cells (16). Most recently, our group demonstrated that MC-modified PEIs can transport into the brain (17,18).

In order to further explore the potential of fatty acid (FA) analogues for brain gene delivery and to understand mechanism(s) of such a FA-based brain gene delivery strategy, a series of FA analogues were conjugated with PEI in the present study. Brain targeting ability and gene transfection effect of these FA-PEI conjugates were investigated.

MATERIALS AND METHODS

Materials

Polyethylenimine (PEI, branched, MW 25 kDa), 6-aminohexanoic acid (EACA), myristoyl chloride, octanoyl chloride, lauroyl chloride, palmitoyl chloride, stearoyl chloride, IR820, rhodamine B isothiocyanate (RITC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4,6-diamidino-2-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR, USA). EDC·HCl was purchased from Gil Bio (Shanghai, China). All the other chemicals were analytical reagent grades. Plasmid pEGFP purchased from Clontech (Palo Alto, CA, USA). Both pEGFP and plasmid glial cell derived neurotrophic factor (pGDNF) were purified using QIAGEN Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Briefly, plasmid DNAs was isolated and purified from *E. coli* based on a modified alkaline lysis procedure by removing RNA, proteins, dyes and low molecular weight impurities in an anion-exchange resin. The purity of DNA was evaluated using electrophoresis on a 1% agarose gel.

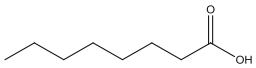
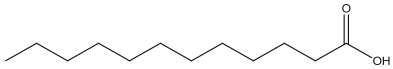
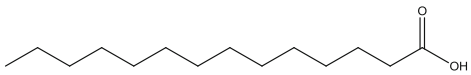
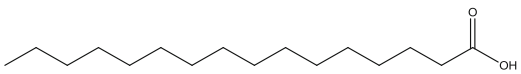
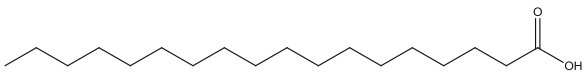
Neuroblastoma cells (SH-SY5Y) were obtained from ATCC (USA) and cultured in a special Dulbecco's modified Eagle medium (DMEM, Gibco Co., USA) supplemented with 10% fetal bovine serum (FBS, Gibco Co., USA).

ICR mice (female, 20–25 g) were obtained from the Experimental Animal Center of Fudan University and maintained under standard housing conditions. All of the animal experiments were carried out according to the guidelines evaluated and approved by the ethics committee of Fudan University (Shanghai, China).

Synthesis of Fatty Acid-PEI Conjugates (FA-PEIs)

A series of FA-PEIs, including octanoic acid (OC)-PEI_{25k}, lauric acid (LC)-PEI_{25k}, MC-PEI_{25k}, palmitic acid (PC)-PEI_{25k}, and stearic acid (SC)-PEI_{25k} (Table I), were synthesized as previously reported (17,18). Briefly, fatty acid chlorides and PEI_{25k} were dissolved in anhydrous DMF separately. Then, 1 ml of fatty acid chloride solutions (*ca.* 0.074 mmol) were added dropwise into 5 ml of PEI_{25k} solutions (*ca.* 0.037 mmol) and stirred overnight at room temperature under a nitrogen atmosphere. Reaction mixtures were centrifuged (12,000 rpm, 4°C) and cold ether was added into supernatants to precipitate crude FA-PEIs. The crude FA-PEIs were purified on an AKTA explorer 100 system (Amersham Biosciences, Uppsala, Sweden) equipped with a Sephadex G25 gel column using distilled water as mobile phase. Finally, the FA-PEIs were obtained through lyophilization and kept at 4°C for further use. ^{1}H-NMR (500 MHz, D_2O) results of FA-PEI products showed a similar peak at 2.5–3.0 ppm, a characteristic peak of -

Table I Library of Synthesized FA-PEIs

Conjugate	Fatty acid	Hydrophobic moiety
OC-PEI $_{25k}$	 octanoic acid	C8
LC-PEI $_{25k}$	 lauric acid	C12
MC-PEI $_{25k}$	 myristic acid	C14
PC-PEI $_{25k}$	 palmitic acid	C16
SC-PEI $_{25k}$	 stearic acid	C18

CH_2CH_2NH- group of the PEI_{25k}, and a peak at 1.3–1.4 ppm, a characteristic peak of -CH_2- backbone of the FA analogues.

Agarose Gel Retardation Assay

To investigate if the FA-PEIs are capable of condensing and delivering DNA, an agarose gel retardation assay was performed (19). An equal volume of pEGFP solution (100 μg/ml) was added into the FA-PEIs solution at a molar ratio of PEI nitrogen (N) to DNA phosphate (P) (N/P) of 6 and incubated for 10 min to prepare FA-PEIs/pEGFP nanoparticles (20,21). Then the FA-PEIs/pEGFP nanoparticles were electrophoresised through a 0.8% agarose gel at 100 V for 25 min and visualized on a UV transilluminator, using naked pEGFP and PEI/pEGFP nanoparticles (N/P ratio: 6) as controls.

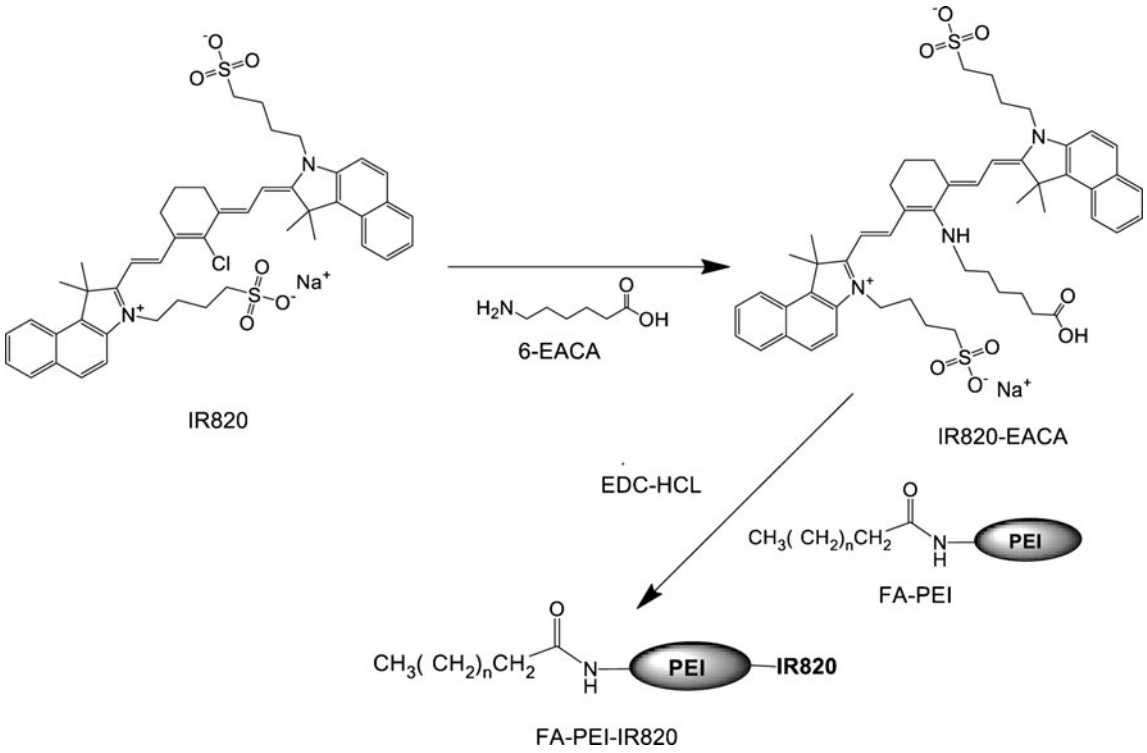
Fluorescence Labeling of FA-PEI_{25k} Conjugates and PEI_{25k}

In order to synthesize fluorescence-labeled FA-PEI_{25k} conjugates and PEI_{25k}, a small molecule, 6-EACA, was conjugated with a NIR fluorescence probe IR820 as reported previously (17,22). Fluorescence labeling of the FA-PEI_{25k} conjugates and PEI_{25k} were performed through coupling 6-EACA-IR820 with the FA-PEI_{25k} conjugates and PEI_{25k} as shown in Scheme 1. Briefly, a solution of the FA-PEI_{25k} conjugates or PEI_{25k} in anhydrous DMF (0.0074 mmol) was added into a mixture solution of EDC·HCl and 6-EACA-IR820 (0.04 mmol) in anhydrous DMF. The mixture was then reacted for 18 h at room temperature and protected from light. The resultant precipitates were washed with cold ether and vacuum dried. Amicon® Ultra-4 centrifugal filters (MWCO, 10 kDa) were used to purify the IR820-labled FA-PEI_{25k} conjugates and PEI_{25k}. Final products were lyophilized and kept at 4°C for further use. ^{1}H-NMR (500 MHz, D_2O) spectra of FAs-PEI-IR820 and PEI_{25k}-IR820 showed chemical shift peaks at 2.1–2.2 ppm belong to the IR820, while peaks at 2.5–3.0 ppm and 1.3–1.4 ppm were characteristic peaks of the FA-PEI_{25k} conjugates.

In Vivo and *Ex Vivo* Targeting Effect of the FA-PEI_{25k} Conjugates

To investigate the brain targeting ability of the FA-PEI_{25k} conjugates, 24 mice were randomly divided into eight groups. 100 μl of saline, IR820, PEI_{25k}-IR820, OC-PEI_{25k}-IR820, LC-PEI_{25k}-IR820, MC-PEI_{25k}-IR820, PC-PEI_{25k}-IR820, and SC-PEI_{25k}-IR820 (1 mg/ml in saline solution) were intravenously (*i.v.*) injected into different group. *In vivo* imaging was performed at 48 h post-injection using an *In Vivo* Imaging System (FX Pro, Kodak, USA) equipped with IR820 filter sets (excitation/emission, 730/790 nm). NIR fluorescence images and optical images were fused together using a Kodak Molecular Imaging Systems software V5.0.1.

Ex vivo imaging studies were subsequently performed to further demonstrate brain distribution behaviors of the IR820-labeled FA-PEI_{25k} conjugates. Briefly, 100 μl of



Scheme 1 Synthesis of FA-PEI-IR820 conjugates.

saline, OC-PEI_{25k}-IR820, LC-PEI_{25k}-IR820, MC-PEI_{25k}-IR820, PC-PEI_{25k}-IR820, and SC-PEI_{25k}-IR820 (1 mg/ml in saline solution) were administrated to six groups of mice, separately. At 0.5, 4, 24, and 48 h following administration, three mice of each group were exsanguinated and brains were excised and fixed with 10% paraformaldehyde. The brains were directly placed on the *In Vivo* Imaging System for imaging.

Brain Uptake of FA-PEI_{25k}/DNA Nanoparticles

To investigate brain uptake of FA-PEI_{25k}/DNA nanoparticles (except for LC-PEI_{25k} due to its poor brain targeting performance), a model plasmid pGDNF was labeled with a fluorescence probe, RITC. Briefly, pGDNF (1 mg/ml) and RITC (1 mg/ml) were mixed in 0.2 M sodium carbonate buffer (pH 9.7) and stirred at 4°C for 12 h. Reaction mixture was then loaded on the Sephadex G25 column and purified using distilled water as an elution medium. RITC-labeled DNA (RITC-pGDNF) was obtained by 70% ethanol precipitation and stored at −4°C avoiding light.

FA-PEI_{25k} conjugates/RITC-pGDNF and PEI_{25k}/RITC-pGDNF nanoparticles (N/P ratio: 6) were prepared as described above. Particle size and zeta potential of PEI/RITC-pGDNF nanoparticles were evaluated using dynamic light scattering (DLS, Malvern, Nano-ZS90). To investigate *in vivo* brain uptake of these nanoparticles, 100 μl of these nanoparticles were injected to mice through tail-vein injection (40 μg DNA/mouse). At 0.5, 12, and 24 h post-administration, three mice of each group were anesthetized with 10% chloral hydrate and then sacrificed by exsanguination. Mouse brains were dissected and visualized using the *In Vivo* Imaging System equipped with RITC filter sets (excitation/emission, 550/620 nm). Mice treated with saline solution were used as a control group.

In Vitro Gene Transfection Study

An equal volume of pEGFP solution (100 μg/ml) was added to the ideal FA-PEI_{25K} and PEI_{25K} solutions in PBS (pH 7.4) at N/P ratios of 6, 8, 12 and 20, separately. Then the mixtures were vortexed for 30 s and incubated for 20 min at room temperature to prepare the FA-PEI_{25k}/pEGFP and PEI_{25k}/pEGFP nanoparticles.

For *in vitro* gene transfection, SH-SY5Y cells were trypsinized and seeded at a density of 1×10^5 cells/well in 24-well plates and incubated for 24 h. The FA-PEI_{25K}/pEGFP or PEI_{25K}/pEGFP nanoparticles (containing 4 μg pEGFP) were added to wells and incubated for 4 h, respectively. Then transfection agents were replaced with 1 ml of fresh culture medium and further cultured for 48 h. The cells were visualized under a fluorescence microscope (IX71, OLYMPUS, Japan). For quantitative study of *in vitro* gene expression, the SH-SY5Y cells were trypsinized and centrifuged at 1,600 rpm for 5 min and cell pellets were resuspended in PBS and analyzed *via* a flow cytometer (BD, USA).

Gene transfection effect of the PEI/DNA nanoparticles was further studied using confocal microscopy. The SH-SY5Y cells were trypsinized and seeded on collagen-coated glass coverslips in 24-well plates at a density of 5×10^4 cells/well and incubated for 24 h. The cells were treated with 1 ml of the FA-PEI_{25K}/pEGFP or PEI_{25K}/pEGFP nanoparticles (N/P ratio: 12, containing 4 μg pEGFP). After 4 h incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. Samples were then washed, counterstained with DAPI and mounted on microslides. The cells were then imaged using a confocal microscope (SP, Leixa, Germany).

In Vivo Gene Transfection Study

In vivo gene transfection study of the PEI/DNA nanoparticles was carried out through multiple injections since this regimen can overcome the elimination or degradation of DNA *in vivo* (23). Briefly, the FA-PEI_{25K}/pEGFP or PEI_{25K}/pEGFP nanoparticles (N/P ratio: 12) were injected *via* mouse tail veins at a dose of 40 μg DNA/mouse/day for four consecutive days. Mice treated with saline solution were used as a control group. The mice were anesthetized with 10% chloral hydrate at 24 h following the last administration, exsanguinated by saline solution and fixed using 4% paraformaldehyde. Brains were excised and fixed in 4% paraformaldehyde for 48 h. Then the brains were treated with sucrose solution and frozen at 80°C in OCT embedding medium (Sakura, Torrance, CA, USA). A cryotome Cryostat (Leica, CM 1900, Germany) was used to cut the frozen brains into 20-μm thick sections. The brain sections were imaged using the Kodak *In Vivo* Imaging System with EGFP filter sets (excitation/emission, 490/520 nm). In order to demonstrate gene expression in different brain regions, the brain sections were stained with 300 nM DAPI for 10 min at room temperature and mounted by glycerol phosphate, then observed *via* the confocal microscope (24,25).

RESULTS

Agarose Gel Retardation Assay

As shown in Fig. 1 (lines 2 to 7), pEGFP was fully retarded for PEI_{25k} and all the FA-modified PEI_{25k} conjugates at a N/P ratio of 6. This result indicated that the hydrophobic modification on the PEI backbone using the FAs did not have interference with the formation of PEI/DNA polyplex, which could be due to the strongly basic nature of PEI as previously reported (26). Therefore, the FA-PEI_{25k} conjugates can be used to condense and deliver DNA.

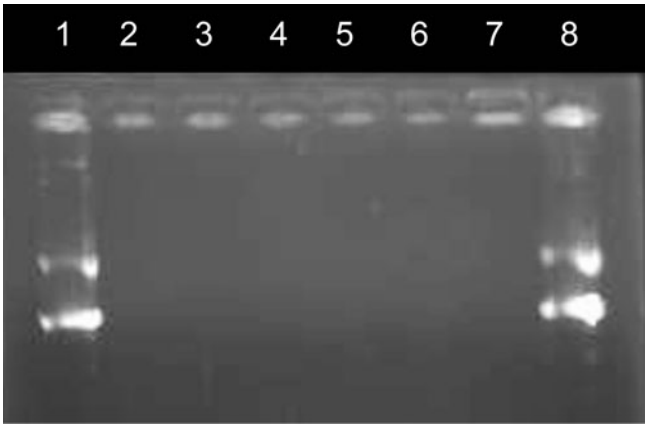


Fig. 1 Agarose gel electrophoresis assay (agarose 0.8%, Hoechst staining) of pEGFP (lanes 1 and 8), PEI_{25k}/DNA (lane 2), OC-PEI_{25k}/DNA (lane 3), LC-PEI_{25k}/DNA (lane 4), MC-PEI_{25k}/DNA (lane 5), PC-PEI_{25k}/DNA (lane 6), and SC-PEI_{25k}/DNA (lane 7).

In Vivo and *Ex Vivo* Targeting Effect of the FA-PEIs

Figure 2 shows the NIR *in vivo* images of mice treated with different IR820 labeled FA-PEI_{25k} conjugates and PEI_{25k} at 48 h post-administration. No NIR fluorescent signals were observed from the brains treated with the IR820 and PEI_{25k}-IR820. All the FA modified PEI_{25k}-IR820 groups showed NIR fluorescent signals in the mouse brains. It was noted that the MC-PEI_{25k}-IR820 group showed stronger fluorescent signal in the mouse brains than other groups at 48 h post-administration.

Ex vivo NIR fluorescent imaging studies were used to investigate the brain distribution behaviors of the FA-PEI_{25k} conjugates (Fig. 3). It can be seen in Fig. 3 that NIR fluorescent signals were observed for all the groups studied during a period of 48 h. Fluorescent intensity of the MC-PEI_{25k}-IR820 group increased gradually with time and maintained high fluorescent signal at 48 h, while that of other FA modified PEI_{25k}-IR820 groups decreased after 24 h. These results indicated that hydrophobic alkyl chain of the FA analogues could mediate PEI to cross the BBB and transport into the brain. In addition, the length of alkyl chain of these FA analogues can affect brain distribution behaviors of these FA-PEIs conjugates. The MC-PEI_{25k} with a 14-carbon moiety showed slower accumulation and thereby could potentially achieve sustained brain targeted delivery.

Brain Uptake of FA-PEI_{25k}/DNA Nanoparticles

Despite the alkyl chain length was different, the FA-PEI_{25k}/DNA nanoparticles had similar particle size approx. 217 nm, which was comparable with that of the PEI_{25k}/DNA nanoparticles (203±15 nm). Moreover, the FA modification on the PEI decreased zeta potential of the

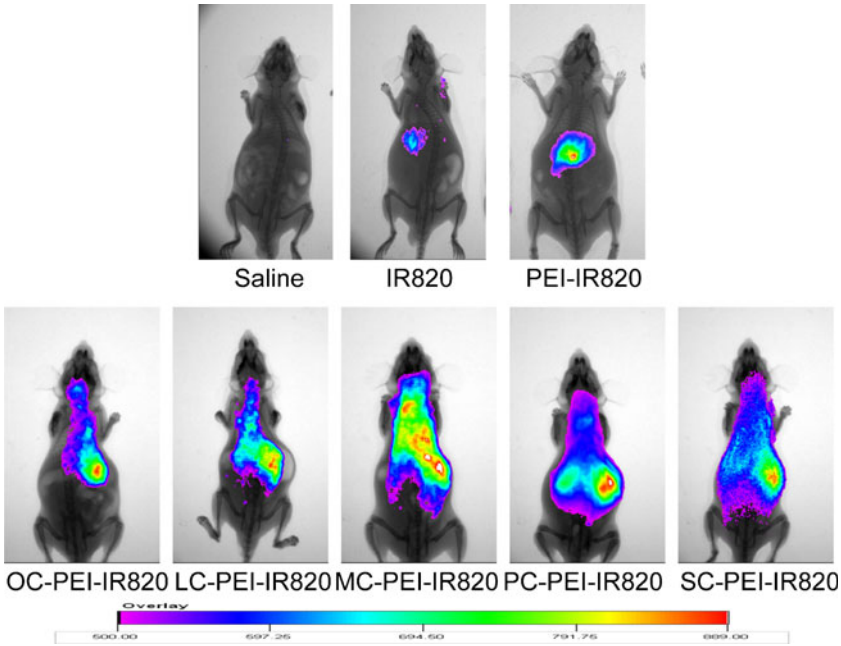


Fig. 2 *In vivo* imaging of mice treated with IR820, PEI_{25k}-IR820, and a series of FAs-PEI_{25k}-IR820 (1 mg/ml in saline solution) 48 h following *i.v.* administration. Mice injected with saline were used as control. *Color bar* on the bottom indicates the signal efficiency of NIR fluorescence emission coming out from animals.

PEI_{25k}/DNA nanoparticles from 28.5±2.1 mv to approx. 21.6 mv and no significant difference was observed for different FA analogues (data not shown).

Figure 4 shows brain uptake of FA-PEI_{25k}/RITC-pGDNF and PEI_{25k}/RITC-pGDNF nanoparticles (N/P ratio=6). No RITC signals were observed in the brains from both the control group and the PEI_{25k}/RITC-pGDNF nanoparticle group. RITC signal in the brains from the FA-PEI_{25k}/RITC-pGDNF nanoparticle group increased gradually with time. The FA-PEI_{25k}/RITC-pGDNF nanoparticles with longer alkyl chain length (i.e. C14, C16, and C18) showed better brain uptake compared to the FA-PEI_{25k}/RITC-pGDNF

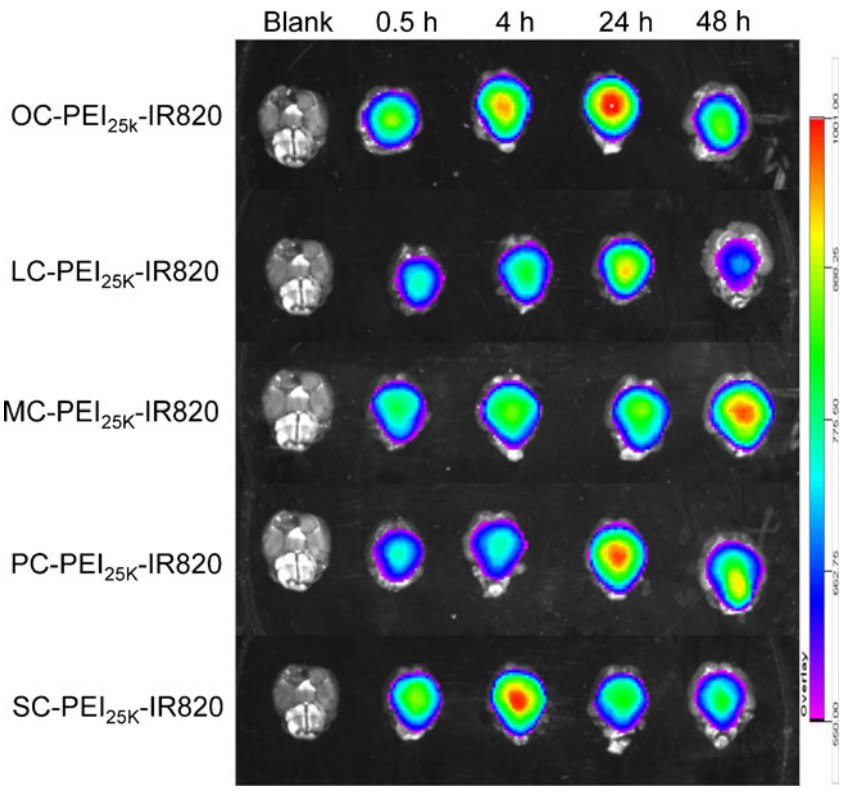


Fig. 3 *Ex vivo* imaging of excised brains from mice treated with OC-PEI_{25k}-IR820, LC-PEI_{25k}-IR820, MC-PEI_{25k}-IR820, PC-PEI_{25k}-IR820, and SC-PEI_{25k}-IR820 at 0.5, 4, 24, and 48 h post-administration. Excised brains from mice treated with saline were used as controls (*Blank*). *Color bar* on the right indicates the signal efficiency of the fluorescence emission coming out from brains.

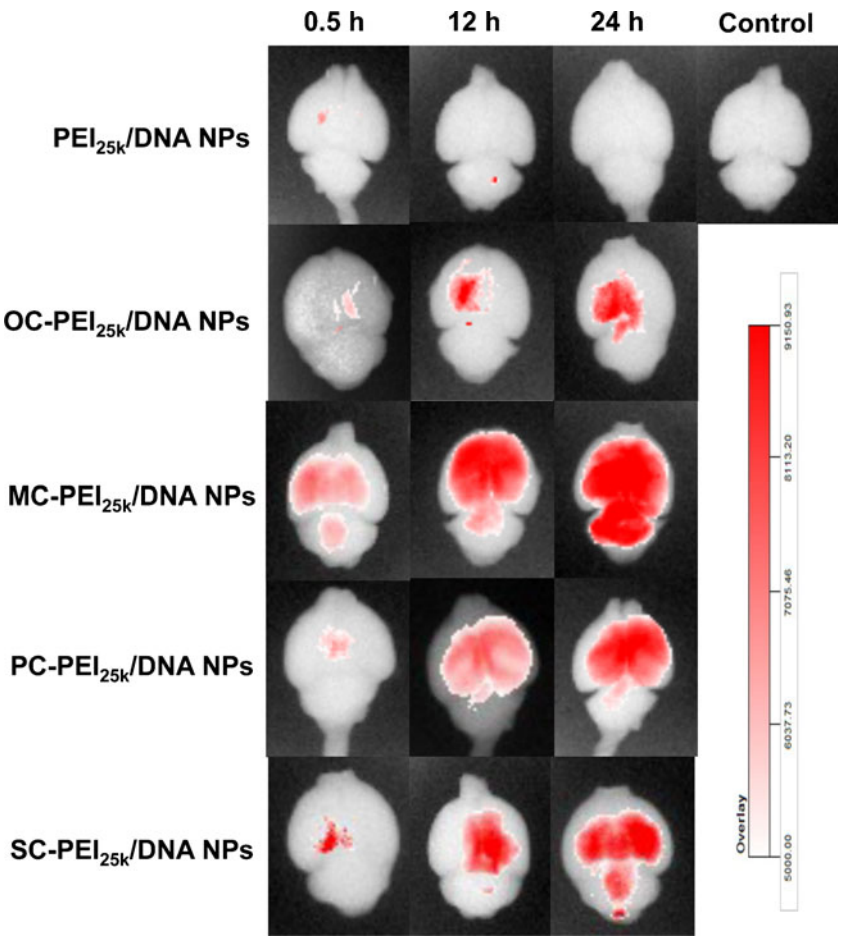


Fig. 4 *In vivo* brain uptake of OC-PEI_{25k}/RITC-pGDNF, MC-PEI_{25k}/RITC-pGDNF, PC-PEI_{25k}/RITC-pGDNF, SC-PEI_{25k}/RITC-pGDNF, and PEI_{25k}/RITC-pGDNF nanoparticles (N/P ratio = 6) at 0.5, 12, and 24 h post-administration. Excised brains from mice treated with saline were used as controls. *Color bar* on the right indicates the signal efficiency of the fluorescence emission coming out from brains.

nanoparticles with shorter alkyl chain length (i.e. C8). Among all FA-PEI_{25k}/RITC-pGDNF nanoparticles studied, the MC-PEI_{25k}/RITC-pGDNF nanoparticles showed strongest RITC signal at different time points. These results suggested that the FA-PEI_{25k} could be a promising brain gene delivery platform. Notably, the MC-PEI_{25k} obtained better brain DNA uptake among all these FA-PEI_{25k} conjugates investigated.

In Vitro Gene Transfection

In vitro gene transfection of the MC-PEI_{25k} was subsequently studied as it showed better brain DNA uptake than other FA analogues. As shown Fig. 5, successful expression of green fluorescent protein in the SH-SY5Y cells was obtained for both the MC-PEI_{25k}/pEGFP and PEI_{25k}/pEGFP nanoparticles. Flow cytometry study showed that EGFP expression increased with the increasing of the N/P ratio. At low N/P ratios (i.e. N/P ratio = 6, 8, and 12), the MC-PEI_{25k}/pEGFP nanoparticles showed significant higher gene transfection compared with the PEI_{25k}/pEGFP nanoparticles ($p < 0.05$). At a high N/P ratio of 20, there was no significant difference in gene transfection between these two groups. There were cell fragments and cell apoptosis observed in the case of PEI_{25k}/DNA nanoparticles at a N/P ratio of 20 (data not shown), while no cytotoxicity was observed for the MC-PEI_{25k}/DNA nanoparticles at the same N/P ratio. These results indicated that the hydrophobic modification on the PEI could reduce its cytotoxicity. The N/P ratio of 12 was used for further studies.

Confocal microscopy was used to localize EGFP expression in the SH-SY5Y cells. It can be seen in Fig. 6 that the MC-PEI_{25k}/EGFP nanoparticles showed better gene transfection effect compared with PEI_{25k}/EGFP nanoparticles (N/P ratio = 12). In addition, the EGFP expression of the MC-PEI_{25k}/DNA nanoparticles localized mainly in the nucleus (blue structures), while the EGFP expression of the PEI_{25k}/DNA nanoparticles was observed in both the cytoplasm and nucleus.

In Vivo Gene Transfection

As shown in Fig. 7c, successful EGFP expression the MC-PEI_{25k}/pEGFP nanoparticles groups was achieved *in vivo*. No EGFP expression was observed in the brain sections of mice

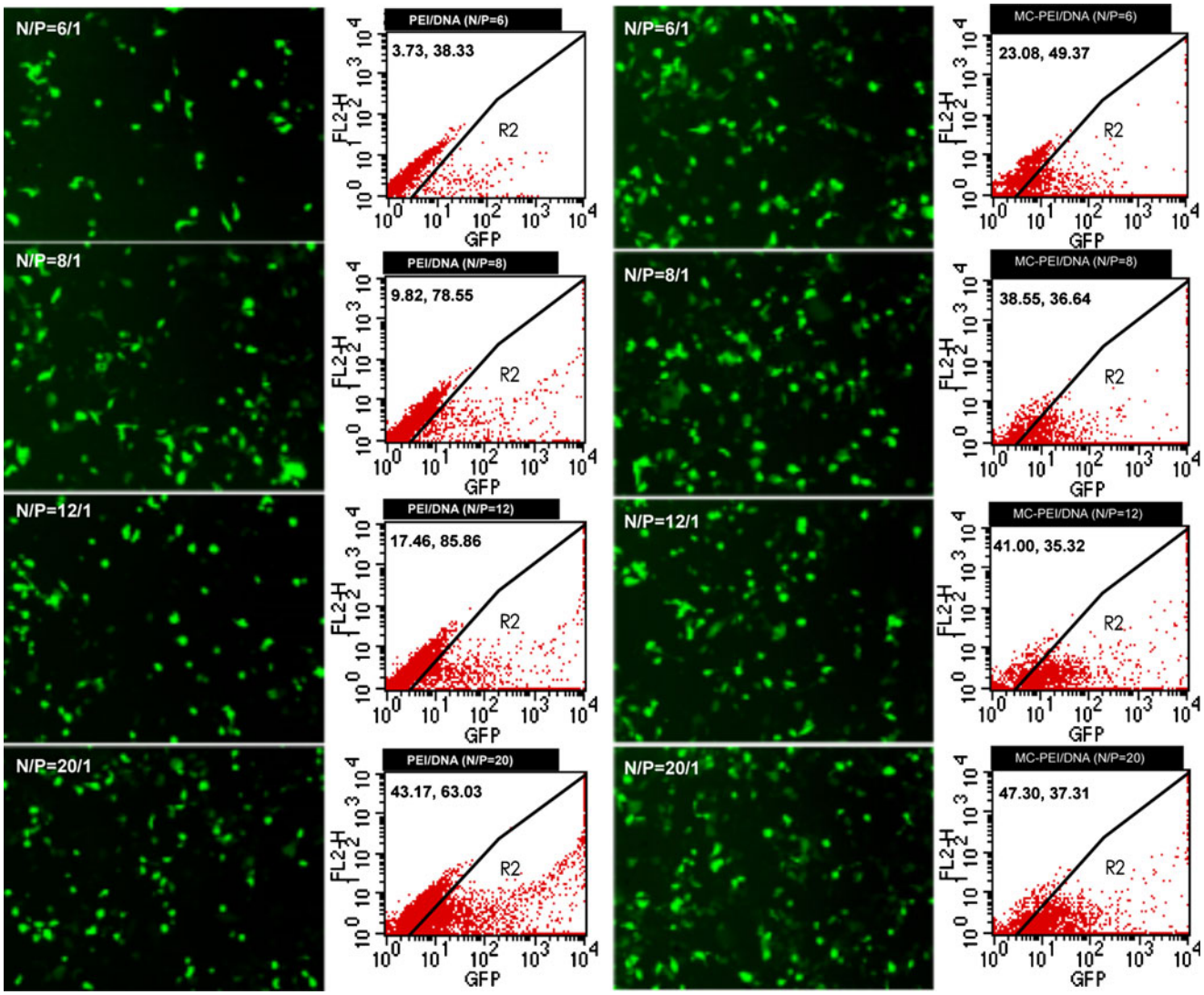


Fig. 5 *In vitro* gene transfection of PEI_{25k}/pEGFP and MC-PEI_{25k}/pEGFP nanoparticles at N/P ratios of 6, 8, 12 and 20. (*Left panel*) PEI_{25k}/DNA nanoparticles; (*Right panel*) MC-PEI_{25k}/DNA nanoparticles.

treated with saline solution (Fig. 7a) or the PEI_{25K}/pEGFP nanoparticles (Fig. 7b). Confocal microscopy studies further confirmed that the expression of green fluorescent protein can be observed in different brain regions, including cortical layer (Fig. 8a-c), hippocampus (Fig. 8a-h) as well as striatum (Fig. 8a-s) for the MC-PEI_{25k}/pEGFP nanoparticles group.

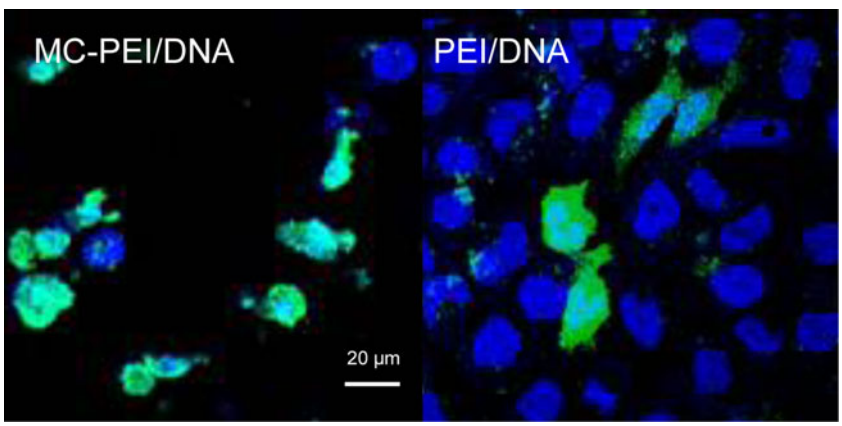


Fig. 6 Confocal microscopy of MC-PEI_{25k}/pEGFP and PEI_{25k}/pEGFP nanoparticles (N/P ratio = 12). Nuclei (*blue*) were stained with DAPI, and the scale bar for each imaging is 20 μm.

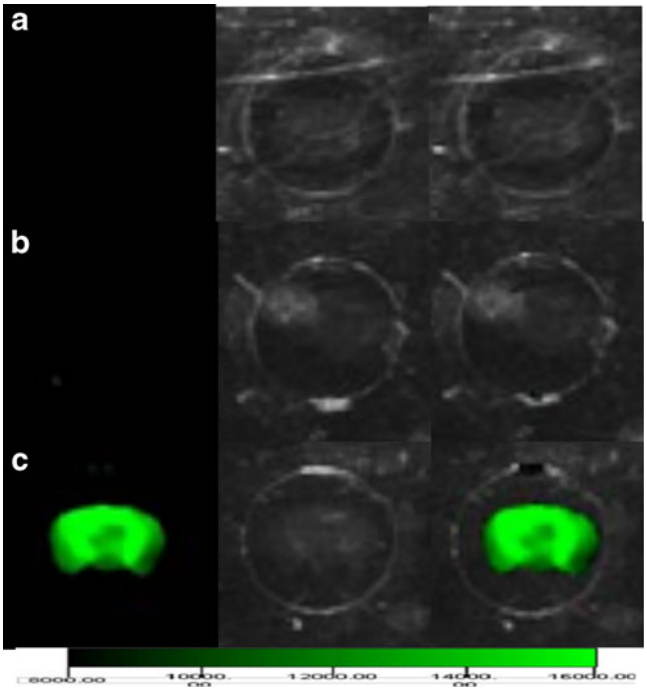


Fig. 7 Fluorescent imaging of brain sections transfected with (**a**) saline; (**b**) PEI_{25k}/pEGFP nanoparticles (N/P ratio = 12); and (**c**) MC-PEI_{25k}/pEGFP nanoparticles (N/P ratio = 12). (*Left*) Fluorescent image. (*Center*) Transmitted light image. (*Right*) Overlay.

No green fluorescent protein expression was observed for PEI_{25k}/pEGFP nanoparticles groups.

DISCUSSION

Non-viral gene delivery to the CNS, especially into the brain, has shown promising for the treatment of severe brain diseases. Among all the non-viral gene-transfer carriers, PEI is one of the most successful and efficient polymeric carriers (27,28). However, PEI has several disadvantages such as high cytotoxicity. Moreover, the PEI itself is not capable of carrying gene therapeutics across the BBB and achieving effective brain gene transfection.

In order to overcome barriers that limit PEI to obtain efficient brain gene delivery, different strategies have been extensively studied. For example, brain targeted ligands (such as RVG and cyclic arginine-glycine-aspartic (RGD)) were conjugated with PEG-PEI to deliver PEI/DNA complexes into the brain (14,29). In addition, hydrophobic small molecules (such as myristic acid and p-hydroxybenzoic acid) were used to modify PEI to achieve brain-targeted delivery (17,18,30).

MC was initially used to modify proteins to improve protein-membrane binding and protein-protein interactions (31). In addition, MC was utilized to modify polyarginines to achieve remarkable cellular internalization and BBB penetration (15,16). It was our assumption that hydrophobic alkyl group of FA analogues might be able to improve membrane permeability of PEI/DNA nanoparticles, thus facilitating BBB penetration of the PEI/DNA nanoparticles. On the other hand, introducing hydrophobic such as alkyl chain might shield high positive charge density on the primary amino groups of PEI backbone, thus eventually decreasing cytotoxicity of the PEI (32,33). Our previous studies demonstrated successful brain targeting and gene delivery mediated by the MC-PEI conjugates (13,14). That was the "proof of principle" of our assumption.

In the current study, a series of fatty acids were conjugated with PEI_{25k} to further investigate this small molecule-based brain gene delivery strategy. As expected, the hydrophobic modification *via* the FAs did not compromise the ability of PEI_{25k} to condense plasmid DNA, but did alter the physicochemical and biological properties of PEI_{25k}. While PEI_{25k}

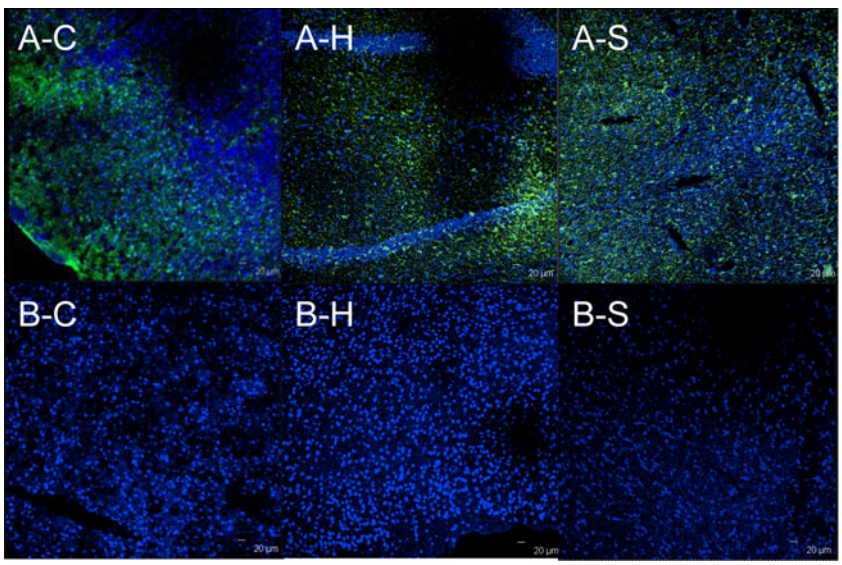


Fig. 8 Confocal microscopy of brain sections of mice treated with (**a**) MC-PEI_{25k}/pEGFP (N/P ratio = 12) and (**b**) PEI_{25k}/pEGFP nanoparticles (N/P ratio = 12). (A-C) and (B-C) cortical layer; (A-H) and (B-H) hippocampus; and (A-S) and (B-S) striatum. Nuclei (*blue*) are stained with DAPI, and the scale bar for each imaging is 20 μm.

itself failed to cross the BBB and mediate brain uptake of its condensed DNA, all the FA-PEI_{25k} conjugates studied were able to penetrate the BBB and obtain successful brain uptake of the condensed DNA. Since the hydrophobicity of these FA-PEI conjugates was different due to different alkyl chain length, the FA-PEI conjugates appeared different brain distribution behaviors. Interestingly, the MC-PEI_{25k} with a medium alkyl length (C14) maintained high brain distribution 48 h post-administration and obtained highest brain DNA uptake among all the FA-PEI_{25k} conjugates investigated. The optimal brain uptake behavior of the MC-PEI_{25k} could be due to the "myristate plus basic" motifs theory (31). More specifically, the hydrophobic interaction mediated by myristate with optimal 14-carbon length might synergize with electrostatic interactions mediated by the PEI to fulfill the BBB penetration through this membrane binding theory. In addition, 14-carbon MC might be able to provide sufficient energy to mediate MC-PEI/DNA nanoparticles to cross the BBB and achieve high brain DNA uptake. Whereas other fatty acids might either not be capable of providing such energy to cross the BBB (such as C8-carbon OC and C12-carbon LC) or form very strong membrane binding with the BBB (such as 16-carbon PC and C18-SC) and thereby overshadow their BBB penetration ability (15). To further confirm the BBB penetration effect of the MC, a low molecular weight PEI (MW1800 Da) was conjugated with the MC. As expected, a similar brain distribution profile was observed for MC-$PEI_{1.8k}$ (data not shown). These results indicated the fatty acid modification of PEI, particularly MC modification, could be utilized to achieve brain targeted gene delivery.

In vitro gene transfection studies showed that MC-modified PEI_{25k} improved gene transfection effect of PEI in the SH-SY5Y cells. The hydrophobic modification of PEI was able to reduce cytotoxicity associated with high cationic charge density of the PEI. In addition, it was noted that the MC modification of the PEI also resulted in different intracellular localization of EGFP expression. The presence of MC on the PEI backbone could possibly change the interaction between PEI and cell membranes or channels and nuclear pores, thus results in difference in the intracellular localization (34). Furthermore, *in vivo* gene transfection and confocal microscopy demonstrated that MC-PEI_{25k} can mediate gene delivery into the global areas of the brain including cortical layer, hippocampus as well as striatum.

Taken together, our data suggest that the fatty acid-mediated brain gene delivery strategy is very versatile and could be used for the treatment of different brain diseases. Currently, work is in progress to further optimize this fatty acid-mediated brain gene delivery platform (including degree of substitution and alkylation position) to maximize its brain gene transfection effect. Meanwhile, other parallel approaches based on MC-modified nanocarriers (such as MC-modified polymeric micelles) are carried out to further explore the potential of this strategy in the field of brain-targeted drug delivery.

CONCLUSION

Fatty acid-based strategies for efficient brain targeted gene delivery were investigated in the present study. These small molecule fatty acids were able to mediate brain-targeted gene delivery. Myristic acid with a medium length of alkyl group showed superior brain DNA uptake among all fatty acid analogues studied. In addition, the myristic acid-modified PEI obtained efficient gene transfection both *in vitro* and *in vivo*. These results indicated that the small molecule fatty acid-modified PEI could be a versatile and efficient brain gene delivery platform for the treatment of a variety of brain diseases.

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REFERENCES

1. Pardridge WM. Brain drug targeting and gene technologies. Jpn J Pharmacol. 2001;87(2):97–103.
2. Schlachetzki F, Zhang Y, Boado RJ, Pardridge WM. Gene therapy of the brain: the trans-vascular approach. Neurology. 2004;62(8):1275–81.
3. Witt J, Marks Jr WJ. An update on gene therapy in Parkinson's disease. Curr Neurol Neurosci Rep. 2011;11(4):362–70.
4. Rogers ML, Rush RA. Non-viral gene therapy for neurological diseases, with an emphasis on targeted gene delivery. J Control Release. 2012;157(2):183–9.
5. Pardridge WM. The blood–brain barrier: bottleneck in brain drug development. NeuroRx. 2005;2(1):3–14.
6. Carvey PM, Hendey B, Monahan AJ. The blood–brain barrier in neurodegenerative disease: a rhetorical perspective. J Neurochem. 2009;111(2):291–314.
7. Pardridge WM. Crossing the blood–brain barrier: are we getting it right? Drug Discov Today. 2001;6(1):1–2.
8. Lewis ME. Crossing the blood–brain barrier to central nervous system gene therapy. Clin Genet. 1999;56(1):10–3.
9. Pardridge WM. Drug and gene delivery to the brain: the vascular route. Neuron. 2002;36(4):555–8.
10. Pardridge WM. Blood–brain barrier delivery of protein and non-viral gene therapeutics with molecular Trojan horses. J Control Release. 2007;122(3):345–8.
11. Perez-Martinez FC, Guerra J, Posadas I, Cena V. Barriers to non-viral vector-mediated gene delivery in the nervous system. Pharm Res. 2011;28(8):1843–58.

12. Lungwitz U, Breunig M, Blunk T, Gopferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm. 2005;60(2):247–66.
13. De Smedt SC, Demeester J, Hennink WE. Cationic polymer based gene delivery systems. Pharm Res. 2000;17(2):113–26.
14. Son S, Hwang do W, Singha K, Jeong JH, Park TG, Lee DS, *et al.* RVG peptide tethered bioreducible polyethylenimine for gene delivery to brain. J Control Release. 2011;155(1):18–25.
15. Pham W, Kircher MF, Weissleder R, Tung CH. Enhancing membrane permeability by fatty acylation of oligoarginine peptides. ChemBioChem. 2004;5(8):1148–51.
16. Pham W, Zhao BQ, Lo EH, Medarova Z, Rosen B, Moore A. Crossing the blood–brain barrier: a potential application of myristoylated polyarginine for *in vivo* neuroimaging. NeuroImage. 2005;28(1):287–92.
17. Meng Q, Yu M, Gu B, Li J, Liu Y, Zhan C, *et al.* Myristic acid-conjugated polyethylenimine for brain-targeting delivery: *in vivo* and *ex vivo* imaging evaluation. J Drug Target. 2010;18(6):438–46.
18. Li J, Gu B, Meng Q, Yan Z, Gao H, Chen X, *et al.* The use of myristic acid as a ligand of polyethylenimine/DNA nanoparticles for targeted gene therapy of glioblastoma. Nanotechnology. 2011;22(43):435101.
19. Masotti A, Pampaloni F. Polyethylenimine bioconjugates for imaging and DNA delivery *in vivo*. Methods Mol Biol. 2011;751:145–65.
20. Ogris M, Steinlein P, Kursa M, Mechtler K, Kircheis R, Wagner E. The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. Gene Ther. 1998;5(10):1425–33.
21. Wagner E, Cotten M, Foisner R, Birnstiel ML. Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci U S A. 1991;88(10):4255–9.
22. Masotti A, Vicennati P, Boschi F, Calderan L, Sbarbati A, Ortaggi G. A novel near-infrared indocyanine dye-polyethylenimine conjugate allows DNA delivery imaging *in vivo*. Bioconjug Chem. 2008;19(5):983–7.
23. Louis MH, Dutoit S, Denoux Y, Erbacher P, Deslandes E, Behr JP, *et al.* Intraperitoneal linear polyethylenimine (L-PEI)-mediated gene delivery to ovarian carcinoma nodes in mice. Cancer Gene Ther. 2006;13(4):367–74.
24. Zanta MA, Boussif O, Adib A, Behr JP. *In vitro* gene delivery to hepatocytes with galactosylated polyethylenimine. Bioconjug Chem. 1997;8(6):839–44.
25. Huang R, Ke W, Liu Y, Jiang C, Pei Y. The use of lactoferrin as a ligand for targeting the polyamidoamine-based gene delivery system to the brain. Biomaterials. 2008;29(2):238–46.
26. Masotti A, Moretti F, Mancini F, Russo G, Di Lauro N, Checchia P, *et al.* Physicochemical and biological study of selected hydrophobic polyethylenimine-based polycationic liposomes and their complexes with DNA. Bioorg Med Chem. 2007;15(3):1504–15.
27. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, *et al.* A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc Natl Acad Sci U S A. 1995;92(16):7297–301.
28. Singha K, Namgung R, Kim WJ. Polymers in small-interfering RNA delivery. Nucleic Acid Ther. 2011;21(3):133–47.
29. Zhan C, Qian J, Feng L, Zhong G, Zhu J, Lu W. Cyclic RGD-poly(ethylene glycol)-polyethyleneimine is more suitable for glioblastoma targeting gene transfer *in vivo*. J Drug Target. 2011;19(7):573–81.
30. Li J, Meng Q, Lei Y, Gu B, Liu Y, Lu W. Benzamide analogue-conjugated polyethylenimine for brain-targeting and gene delivery. J Drug Target. 2011;19(9):814–20.
31. Resh MD. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta. 1999;1451(1):1–16.
32. Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc Natl Acad Sci U S A. 2002;99(23):14640–5.
33. Tian H, Xiong W, Wei J, Wang Y, Chen X, Jing X, *et al.* Gene transfection of hyperbranched PEI grafted by hydrophobic amino acid segment PBLG. Biomaterials. 2007;28(18):2899–907.
34. Kim S, CHoi JS, Jang HS, Suh H, Park J. Hydrophobic modification of polyethyleneimine for gene transfectants. Bull Korean Chem Soc. 2001;22(10):1069–75.