ApoE-2 Brain-Targeted Gene Therapy Through Transferrin and Penetratin Tagged Liposomal Nanoparticles

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Abstract

Purpose—Apolipoprotein E2 (ApoE2) gene therapy is a potential disease-modifying therapy for Alzheimer's disease (AD). We investigated the potential of plasmid encoding ApoE2 loaded brain-targeted functionalized-liposomes for treatment of AD. This was achieved *via* systemic administration of liposomes entrapping therapeutic gene targeting the brain of mice.

Methods—Targeting and transfection efficiency of designed liposomes were determined in bEnd.3, primary glial and primary neuronal cells. The ability of liposomal formulations to translocate across *in vitro* blood-brain barrier (BBB) and, thereafter, transfect primary neuronal cells was investigated using *in vitro* triple co-culture BBB model. We quantified ApoE expression in the brain of mice after single intravenous injection of brain-targeted liposomes loaded with plasmid ApoE2.

Results—Dual surface modification enhanced the *in vitro* transfection efficiency of designed liposomes. Successful delivery of therapeutic gene overcoming BBB by Transferrin-Penetratin-modified liposomes was demonstrated both *in vitro* and *in vivo*. Significant ($p < 0.05$) increase in ApoE levels in the brain of mice was observed after intravenous administration of Tf-Pen-liposomes encasing plasmid ApoE2.

Conclusion—The results indicate that dual-ligand based liposomal gene delivery systems had both enhanced brain targeting and gene delivery efficiencies. Transferrin-Penetratin modified liposomes for delivery of plasmid ApoE2 has great potential for AD treatment.

Keywords

ApoE2; gene therapy; liposome; penetratin; transferrin

INTRODUCTION

Alzheimer's disease (AD), specially the late onset form of AD (LOAD), is the common cause of dementia in individuals age 60 and older (1). This age-dependent neurological disorder is characterized by the presence of extracellular amyloid plaques composed of amyloid-β (Aβ) and intracellular neurofibrillary tangles (2,3). Mounting evidence supports

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the role of Aβ aggregation and accumulation as an early trigger of toxic cascade leading to synaptic dysfunction, neurodegeneration and cognitive impairment in the etiology of AD (4,5). Multiple genetic and environmental risk factors are involved in LOAD pathogenesis. Impairment of Aβ clearance is probably the major contributor to disease development. While the ε4 allele for APOE gene is the strongest genetic risk factor amongst its three polymorphic alleles (ε2, ε3 and ε4), the ε2 allele is protective (6-8). Many lines of evidence have shown that the risk factors of AD (aging and APOE ε4) accelerate accumulation of Aβ prior to the development of the disease. The apolipoprotein E (ApoE), in the central nervous system (CNS), transports cholesterol from astrocytes to neurons through cell surface receptors, including the low density lipo-protein receptor (LDLR) and LDLR-related protein 1 (LRP1) (9,10). Consequently, ApoE contributes to synaptic plasticity and neuronal function by controlling cholesterol homeostasis with the ApoE3 and ApoE2 alleles having superior function than ApoE4 (11).

A potential therapeutic approach in the development of disease-modifying therapies for AD considers the neuropro- tective functions of APOE ε2 allele. It has been suggested that ApoE2 isoform may differentially regulate Aβ clearance through neurons, microglia or delivery to blood brain barrier (BBB). These features of E2 isoform could be attributed to its higher conformational stability, lower binding affinity to LDLRs, and greater affinity to bind Aβ as compared to E3 and E4 isoforms (11-13). Therefore, ApoE2 isoform expression through targeted therapy may help establish gene based target therapy for AD prevention and treatment.

In this research, we investigated the potential of ApoE2 gene therapy to induce ApoE overexpression in the brain of mice using functionalized-liposomes with ligands-promoter of BBB crossing as gene delivery carriers. Since transferrin (Tf) receptors are expressed on brain capillary endothelial cells (14,15), liposomes were surface modified with transferrin ligand for brain-targeted gene delivery. Additionally, we employed the use of Penetratin (Pen) conjugated to DSPE-PEG-liposomes. Pen (RQIKIWFQNRRMKWKK) is a cell-penetrating peptide that plays important role on enhancement of translocation across cellular membrane of their associated cargo including Pen-associated liposomes (16,17). The current study shows the efficiency of Tf-Pen conjugated liposomes encapsulating chitosan-plasmid ApoE2 complexes to deliver pApoE2 to the brain of mice and induce the production of ApoE2 as a new therapeutic approach for treatment of AD.

MATERIAL AND METHODS

Synthesis of DSPE-PEG$_{2000}$-Pen and DSPE-PEG$_{2000}$-Tf

For preparation of liposomal formulations, first we coupled Pen and Tf to terminal NHS-activated DSPE-PEG$_{2000}$ phospholipid, separately. Briefly, the Pen and DSPE-PEG$_{2000}$-NHS (1:5 M ratio) were dissolved in anhydrous DMF, and adjusted the pH to 8.0–8.5 with triethylamine. After 120 h, uncoupled Pen was removed by dialysis (molecular weight cut-off of 3500 Da) against deionized water for 48 h. The resultant product was lyophilized and stored at −20°C until use. Holo-Tf (Sigma-Aldrich, St. Louis, MO, USA) and DSPE-PEG$_{2000}$-NHS (125 μg Tf/μM phospholipid) were dissolved in anhydrous DMF, adjusted pH to 8.0–8.5 with triethylamine, and stirred for 24 h at room temperature. The product was

passed through Sephadex G-100 column to removed unbound protein. Coupling efficiencies of both reactions were determined using BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA).

Preparation and Characterization of Liposomes

Pen-PEG-lipid (4 mol%), DOPE (45 mol%), DOTAP (45 mol%), and Cholesterol (2 mol%) were combined in chloroform:methanol (2:1, v/v) and dried to form a thin lipid film, which was thereafter hydrated using HEPES buffer (pH 7.4). Pen-liposomes were formed after sonication of the sample. Pen-liposomes were stirred overnight with Tf-micelles (4 mol%) to form Pen-Tf-liposomes. The liposomes were passed through Sephadex G-100 column to remove free Tf, and then, extruded through polycarbonate membrane (0.2 μm). For preparation of chitosan-pDNA complexes, 1% *w/v* chitosan (MW 30 kDa) was dissolved in 0.2 M acetate buffer (pH 4.5). The pDNA solution was added to chitosan at N/P ratio of 5 and stirred for 30 min. Thereafter, chitosan-pApoE2 polyplexes were added to the hydration buffer for incorporation into liposomes. Hydrodynamic size and zeta potential of the formulations were determined by dynamic light scattering using Zetasizer Nano ZS 90 (Malvern Instruments, Malvern, UK) at 25°C. Percent encapsulation of plasmid ApoE2 into liposomes was calculated using Hoechst 33342 (0.15 μg/mL), 354/458 nm excitation/ emission wavelengths, respectively, considering absorbance in presence of 0.5% *v*/v Triton X-100 as 100%.

Chitosan-pDNA Binding Ability

The molar ratios of the amine groups in chitosan and phosphate groups in DNA, chitosan-pApoE2 N/P ratio, were monitored by ethidium bromide (EtBr) exclusion assay and agarose retardation assay. Free pApoE2 was used as a positive control. Different chitosan weight ratios (1-5, 10, 15 and 18) were complexed to pApoE2 (1 μg) and stained with EtBr (0.5 μg) for 5 min. Fluorescence intensity (excitation/emission wavelengths: 260/600 nm, respectively) was measured using a spectrophotometer. For agarose retardation assay, chitosan weight at different ratios (1,5,10,15 and 18) were complexed to pApoE2 (1 μg), then loaded in 0.8% *w/v* agarose gel stained with EtBr (0.5 μg/mL) and electrophoresed at 80 V in 0.5× Tris-acetate-EDTA (TAE, Bio-Rad, CA, USA) buffer for 80 min. The pDNA migration was thereafter recorded.

DNase Protection Assay

Liposomal formulations encapsulating pApoE2 (1 μg) were incubated for 60 min at 37°C with 1-unit DNase I. Addition of 5 μl of EDTA (100 mM) stopped the reaction. Subsequently, 20 μL of heparin (5 mg/mL) was added and the mixture incubated for 2 h at room temperature to release the pDNA from the complex. The released pDNA samples were subjected to agarose gel electrophoresis 0.8% (*w/v*) at 80 V for 80 min. Naked pApoE2 incubated with DNase I was used as a positive control.

Cell Culture and Animals

The mouse brain endothelial cell line bEnd.3, obtained from ATCC (Manassas, VA) were cultured in DMEM 10% *v*/v fetal bovine serum (FBS) and 1% *v*/v Penicillin Streptomycin

(p/s) solution. Primary glial cells and primary neuronal cells were isolated from brain of 1-day old rats. Primary glial cells were cultured in DMEM with 10% v/v FBS and 1% v/v p/s. Glial fibrillary acidic protein (GFAP) immunostaining confirmed the purity of the culture. Primary neuronal cells were cultured in Neurobasal media with 10% v/v plasma-derived horse serum, B-27 supplements, L-Glutamate (25 mM) and 1% v/v p/s. Cytosine arabinoside (10 μM) was added on day 3 to remove non-neuronal cells. Anti-MAP2 antibody immunostaining confirmed the purity of the culture. Cells were incubated in atmosphere of 5% CO_2 at 37°C.

Animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at North Dakota State University. Rats (male/female) were purchased from Charles River Laboratories (Wilmington, MA, USA) and C57BL/6 (male/female) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The animals were housed maintained under standard conditions with free access to food and water and exposed to 12 h light-dark cycle.

In Vitro Transfection Efficiency

Evaluation of transfection efficiency of liposomal formulations containing chitosan-pApoE2 complexes were performed in bEnd.3, primary glial and primary neuronal cells. Cells (1×10^6 cells/well) were seeded in 6-well plates, 24 h prior to experiment. Liposomal formulations (100 nM) containing chitosan-pApoE2 complexes (1 μg) were added to the cells in serum-free medium. After 4 h, the media was replaced and cells incubated for a total of 48 h. ApoE expression was quantified using Apolipoprotein E ELISA kit (Thermo Fisher Scientific). Supernatant and cell lysates were incubated for 2.5 h in 96-well pre-coated plates. Biotinylated antibody was added and incubated for 1 h. HRP reagent was incubated 45 min. Subsequently, TMB substrate was incubated for 30 min, the reaction was stop with stop solution followed by colorimetric measurement at 450 nm and 550 nm. Subtraction of 550 nm values from 450 nm values corrected the optical imperfections in the plate. Measured values were normalized by total protein levels in the sample, which were determined by BCA protein assay.

Design of *In Vitro* Triple Co-Culture BBB Model

The *in vitro* triple co-culture BBB model was designed combining bEnd.3, primary glial and primary neuronal cells. Primary glial cells (1.5×10^4 cells/cm^2) were seeded on the bottom side of culture inserts (0.4 μm pore size and 0.33 cm^2 area) in DMEM with 20% v/v FBS, 1% v/v p/s, overnight incubation. Then, bEnd.3 cells (1.5×10^4 cells/cm^2) were seeded on upper side of culture inserts that were placed in 24-well plates. Measurement of transendothelial electrical resistance (TEER) values using EVOM^2 (World Precision Instruments, Sarasota, FL, USA) evaluated the formation of tight junctions. Primary neuronal cells were cultured in the bottom of 24-well plate.

Transfection Efficiency in Primary Neuronal Cells Following Liposome Transport across *In Vitro* BBB Model

Liposomal formulations (100 nM) encapsulating chitosan-pApoE2 complexes (1 μg) were added to the upper compartment of inserts and incubated for 8 h. Thereafter, the inserts were

removed and the media was replaced for fresh media. After 48 h incubation, the amount ApoE produced by primary neuronal cells was determined by Apolipoprotein E ELISA kit, as described previously. Measured values were normalized by total protein levels in the samples, which were determined by BCA protein assay.

Blood Compatibility Study

In vitro biocompatibility of liposomal formulations was evaluated in freshly harvested erythrocytes from Sprague-Dawley rats. The blood was centrifuged at 1500 rpm for 10 min and washed three times with PBS, pH 7.4, 10 mM CaCl2. Erythrocyte solution containing 1.5×10^7 cells was incubated with negative control (PBS), positive control (Triton X-100 1% *v*/v) or liposomal formulations (31.25–1000 μM) for 1 h at 37°C. Thereafter, samples were centrifuged at 1500 rpm for 10 min. Absorbance of supernatants was measured at 540 nm. Percent hemolysis was calculated considering the absorbance in the presence of Triton X-100 as 100%.

In Vivo Transfection Efficiency and Biocompatibility

For study of transfection efficiency of liposomal formulations containing chitosan-pApoE2 complexes, seven C57BL/6 mice were injected with single dose of either PBS, or pApoE2 (1 mg pApoE2/kg body weight), or Plain-lip or PenTf-liposomes (~15.2 μ moles phospholipids/kg body weight) encapsulating 1 mg pApoE2/kg body weight. After 5 days of administration, different organs (brain, liver, kidneys, heart, lungs, spleen and blood) were removed, weighed, homogenized in RIPA buffer containing proteinase and phosphatase inhibitor cocktail and centrifuged at 4000 rpm, 4°C for 15 min. ApoE2 levels in the samples were quantified using Apolipoprotein E ELISA kit as previously described. Tissue samples from control mice (PBS administration) were similarly processed to quantify the ApoE expression of individual organs. Measured values were normalized by total protein levels in the samples, which were determined by BCA protein assay. Hematoxylineosin (H&E) staining were performed in tissue sections to analyze the tissue characteristics and the possible morphological changes after treatment.

RESULTS

Preparation and Characterization of Liposomes

Conjugation of Pen and Tf to DSPE-PEG2000-NHS were performed as described previously. Coupling efficiency of Pen-PEG lipid reaction was 88.3 ± 1.7% and coupling efficiency of Tf-PEG lipid reaction was 83.3 ± 5.8%. Dynamic light scattering analysis showed particle size range from 144 nm to 166 nm with a relative small size distribution (PDI 0.3), Table I. We observed that dual modification of liposome enhanced the stability of nanoparticle as demonstrated by the smaller hydrodynamic particle size of Tf-Penliposome as compared to the other liposomal formulations and by the TEM images of unmodified-liposome (Fig. 1a) and Tf-Pen-liposome (Fig. 1b). The formulations showed analogous spherical shape and compacted structure. Liposome surface modification had direct influence on zeta potential. The positive charge of the phospholipid DOTAP contributed to positive zeta potential of Liposome (39.4 ± 3.22 mV). While zeta potential of Pen-liposomes was 30.6 ± 1.29 mV. The negative character of Tf significantly ($p < 0.05$)

reduced the zeta potential as compared to that of control Liposome. For Tf-Pen-liposome, the positive charge of Pen balanced the negative charge of Tf leading to zeta potential of 23 $\pm$ 0.23 mV. Efficient encapsulations of plasmid ApoE2 into liposomes was obtained for all formulations (Table I), which were above 90%.

Chitosan-pDNA Binding Affinity and pDNA Protection against Enzymatic Degradation

Chitosan was complexed to pDNA at different N/P ratios and the effect of chitosan on pDNA condensation was investigated using EtBr exclusion assay and gel retardation assay. The results of EtBr exclusion assay indicated that the N/P ratio of chitosan-pDNA greater than 5 led to tight binding of chitosan and pDNA. As depicted in Fig. 2a, the relative fluorescence intensity reduced significantly ($p < 0.05$) from N/P of 1 (97.7 $\pm$ 2.6%) to N/P of 5 (19.6 $\pm$ 3.2%), whereas we did not observe significant reduction of fluorescence intensity from N/P of 5 to N/P of 10, 15 and 20. This suggests that chitosan-plasmid ApoE2 interactions increased upon increasing N/P ratio. Gel retardation assay was used to determine any change in the electrophoretic mobility of complexed pDNA. Chitosan-pDNA complexes were created at N/P ratios of 1, 5, 10, 15 and 20. Uncomplexed pDNA was represented by the bright band in Fig. 2b lane a. Fig. 2 lane b showed the partial condensation of pDNA at N/P of 1. While Fig. 2b lanes c-f demonstrated complete pDNA complexation in chitosan condensates. The gel retardation assay indicates that chitosan has the ability to compact pDNA and the N/P ratio affects the binding degree. Based on our findings, N/P ratio of 5 was chosen for our experiments.

For effective gene expression, gene carriers must be able to overcome nuclease degradation and deliver the nucleic acid into the nucleus of cells (19). In order to evaluate the protective effect of liposomal nanoparticles on pDNA against enzymatic degradation, the formulations loaded chitosan-pApoE2 complexes were challenged in presence of DNase I. Nuclease digestion was assessed by comparing the signal intensity of the treated samples with the control. The integrity of pApoE2 was represented by Fig. 2c lane a. Upon incubation with DNase I, naked pDNA was completely degraded (Fig. 2c lane b). In contrast, DNase protection assay showed that all liposomal formulations protected pApoE2 from enzymatic degradation (Fig. 2c, lanes c-f) with signal intensities similar to control.

In Vitro Transfection Efficiency

For evaluating *in vitro* transfection efficiency, bEnd.3 (Fig. 3a), primary glial (Fig. 3b) and primary neuronal cells (Fig. 3c) were treated with liposomes encapsulating chitosan-pApoE2 complexes. Quantification of ApoE levels after transfection showed the enhanced ability of Tf-Pen-liposomes to significantly ($p < 0.05$) increase ApoE levels as compared to Liposome, Tf-lip, Pen-lip, naked ApoE2 and Lipofectamine 3000/pApoE2 treatment in the aforementioned cells, and cellular ApoE levels (untreated cells).

In Vitro Co-Culture BBB Model

A significant number of potential drugs to brain diseases have failed mainly due to the restrictive permeability of BBB. The characterization of BBB penetration of therapeutic candidates during the development process can have significant impact for the success of the final product (20). Therefore, there is great interest for *in vitro* models that reflect the

properties of the biological barrier. In attempt to mimic the BBB, we designed an *in vitro* co-culture model by culturing brain endothelial (bEnd.3) cells and glial cells on the upper and bottom side of culture inserts, respectively. This model showed significantly ($p < 0.05$) higher TEER (401.5 ± 8.5 Ωcm^2) as compared to TEER of monolayer models of bEnd.3 (191.9 ± 7.1 Ωcm^2) and glial cells (135.7 ± 2.8 Ωcm^2), Fig. 4a. Glial cells are postulated to increase the tightness of endothelial monolayers as reflected by TEER values of co-culture model. Therefore, the *in vitro* co-culture BBB model represents a physiologically-relevant BBB model, which is adequate for functional studies of BBB and selection of brain-targeted formulations (18).

Following the design of *in vitro* co-culture BBB model, we assessed the ability of liposomal formulations loading chitosan-pApoE2 complexes to cross the *in vitro* barrier layer and, subsequently, transfect primary neuronal cells. Liposomal formulations (100 nM) containing chitosan-pApoE2 complexes (1 μg) were added in the inserts, which were placed in the same well with primary neuronal cells cultured in the bottom, for 8 h. TEER values were measured before and after liposomal incubation to evaluate the potential cytotoxicity of formulations to the barrier layer. We did not observe significant differences between TEER values before and after incubation of liposomal formulations (Fig. 4b). This strongly indicates that liposomes did not cause membrane disruption or cellular damage after transport across the *in vitro* BBB model, therefore the integrity of the cellular barrier was maintained throughout the experiment.

Transfection Efficiency in Primary Neuronal Cells Following Liposome Transport across *In Vitro* BBB Model

After transcytosis across *in vitro* BBB model, we evaluated the functionality of designed liposomal formulations through quantification of transfection efficiency in primary neuronal cells. Tf-Pen-liposomes encapsulating chitosan-pApoE2 complexes demonstrated significantly ($p < 0.05$) higher ability to cross the *in vitro* BBB and induce ApoE expression in primary neuronal cells as compared to unmodified liposome, Tf-lip, Pen-liposomes and cellular ApoE levels (Fig. 5). The results suggested that the presence of both ligands on liposome surface determined the improved transport across BBB model as well as transfection in primary neuronal cells.

Blood Compatibility Study

Blood compatibility study is an important parameter to evaluate the biological safety of formulations to be administered intravenously. In this study, we observed that the interaction of nanoparticles with red blood cells caused erythrocyte lysis dependent on liposome phospholipid concentration. Increasing liposomal concentration, the hemolysis percentage increased (Fig. 6). At 31.25 nM phospholipid concentration, we observed in average 1% hemolysis. While at 1000 nM phospholipid concentration, we observed in average 9.5% hemolysis for Liposome, Tf-liposome and Tf-Pen-liposome, and 10.7% hemolysis for Pen-liposome.

In Vivo Transfection Efficiency and Biocompatibility

Our group has previously determined the biodistribution of liposomes modified with transferrin and penetratin in the brain of mice (21). The study compared the ability of liposome without surface modification, Tf-liposome, Pen-liposome and PenTf-liposome to penetrate the BBB, reach brain parenchyma and induce protein expression in brain cells. The results demonstrated that dual-functionalization of liposomes with Tf and Pen significantly ($p < 0.05$) enhanced the accumulation of liposomes in the brain of mice and induced protein expression as compared to the other liposomal formulations (21). Hence, dual-functionalization of liposome was important for brain-targeting. Following to these findings, next we evaluated whether single intravenous administration of Liposome or Tf-Pen-liposomes containing chitosan-pApoE2 (1 mg pApoE2/kg body weight) would significantly increase the levels of ApoE in the brain of mice. We observed that Tf-Pen-liposomes (20.8 ± 4.0 ng/μg of protein) significantly ($p < 0.05$) increased ApoE expression in the brain of mice as compared to unmodified liposomes encapsulating chitosan-pApoE2 complexes (14.4 ± 3.4 ng/μg of protein), naked plasmid ApoE2 (3.4 ± 1.8 ng/μg of protein) and endogenous ApoE levels (2.5 ± 2.0 ng/μg of protein), as depicted in Fig. 7. No significant differences in ApoE levels were observed in liver, kidneys, heart, lungs and spleen when comparing the endogenous ApoE levels and administration of naked ApoE2, Liposome and Tf-Pen-liposomes containing chitosan-pApoE2 complexes. Additionally, we observed that Tf-Pen-liposomes containing chitosan-pApoE2 significantly ($p < 0.05$) increased ApoE protein levels in the blood compared to endogenous protein levels (control group) and administration of plasmid ApoE2.

For evaluation of *in vivo* biocompatibility of liposomal formulations encapsulating chitosan-pApoE2 complexes, tissue sections of major organs such as brain, liver, kidneys, heart, lungs and spleen, were stained with hematoxylin and eosin (H&E), and compared to tissue sections of control (PBS administration). No obvious lesions, signs of inflammation or change in cell morphology were observed in the tissue sections (Fig. 8).

DISCUSSION

APOE gene has been established as the most important genetic risk factor for developing LOAD. The most common variants of ApoE are ApoE4, ApoE3 and ApoE2 (22). Increasing evidence shows that ApoE isoforms have differential roles on AD pathology and they might regulate AD-pathways through Aβ metabolism, however the mechanisms involved are still unclear. ApoE4 allele is known as the most important genetic risk factor for AD, whereas ApoE2 allele is protective (10). ApoE4 could predispose to AD by suppressing Aβ clearance in the brain, and enhancing Aβ oligomerization leading to increase of toxic Aβ oligomers (11,23). ApoE2 could modulate Aβ clearance, reduce Aβ levels and pathogenic plaques more efficiently as compared to ApoE4 (24,25). Despite the major safety concerns involving viral vectors (i.e. immunogenicity, cytotoxicity and insertional mutagenesis), viral gene delivery of ApoE2 through intracerebral administration has demonstrated to markedly reduce Aβ burden in AD mouse model (26). Therefore, ApoE2 gene therapy is a potential disease-modifying therapy for prevention and treatment of AD. In this study, we synthesized

liposome surface modified with Tf and Pen for non-viral brain-targeted delivery of plasmid ApoE2 to the brain of mice using a non-invasive route of administration.

The tight regulation of movement of ions, molecules and cells between the blood and the brain by BBB limits the delivery of therapeutic agents into CNS. The development of properly designed nanocarrier that ensure BBB crossing and delivery of therapeutic gene into brain cells is expected to play important role on AD therapy (27). We assumed that Tf ligand mediates the transport of our designed brain-targeted liposome across BBB through receptor-mediated endocytosis and Pen peptide enhances liposome internalization into cells and assist in overcoming receptor saturation. Hence, we expected that our system would efficiently cross the BBB and deliver plasmid ApoE2 to brain cells followed by induction of protein expression. The rational synthesis of these cationic liposomes consisted of using equimolar ratio of DOPE and DOTAP phospholipids, which would provide enhanced cellular penetration and promotion of transfection (28). Cholesterol was used as stabilizer, controlling the fluidity of liposome bilayer and preventing vesicle aggregation (29). DSPE-PEG phospholipid contributed to improve carrier pharmacokinetics after intravenous administration by minimizing protein interaction, recognition by macrophage and, consequently, reducing nanoparticle clearance with ensuing prolonged systemic circulation (30,31). Finally, Tf ligands were conjugated to liposomes to provide brain-targeted properties, while conjugation of Pen peptide to liposomes would improve their internalization into cells and promote transfection.

Surface modification of liposomes improved the stability of nanoparticles leading to a lower particle size as compared to unmodified liposomes. Additionally, dual-functionalization enhanced this effect. In general, the designed liposomes were characterized by uniform size distribution and positive zeta potential. Plasmid DNA were complexed to the polycationic polymer chitosan as additional strategy for improvement of transfection. Chitosan positive charges electrostatically interact with the negative charges of DNA and spontaneously form chitosan-DNA complexes leading to DNA packing. As a consequence, nucleases are sterically prevented to access and degrade the condensed nucleic acid (32,33). However, a balance in chitosan-pDNA interaction needs to be adjusted to provide, simultaneously, DNA packing and protection against enzymatic degradation, as well as nucleic acid release from the complex once at the target site (33,34). Based on our analysis of chitosan-pDNA binding affinity using EtBr exclusion assay and gel retardation assay, the N/P ratio of 5 was chosen for preparation of chitosan-pApoE2 complexes. Furthermore, highest *in vitro* transfection efficiency has been demonstrated at N/P of 5 for chitosan/plasmid complexes (35,36). DNA molecules are vulnerable to DNase degradation particularly *in vivo* and during long storage. Therefore, to produce effective transfection gene carriers are required to prevent enzymatic degradation of the nucleic acid before delivering the genetic material into the cells. Our findings showed that the liposomal system were able to protect pApoE2 under the presence of nucleases.

Poor targeting property is a common limitation faced by liposome-based targeted gene delivery systems, which is demonstrated by negligible penetration at the target site and accumulation in the liver. The design of targeting ligands has been progressing and showing to improve the capability of carriers to deliver nucleic acids to desired cells and,

consequently, enhance the transfection (19,21,37,38). Studying the transfection efficiency of designed liposomes loading chitosan-pApoE2 complexes, we observed that the combination of brain-targeted ligand (Tf) and cellular penetration enhancer (Pen) on liposome surface were actively involved in the successful *in vitro* transfection of bEnd.3, glial and primary neuronal cells.

The efficacy of treatment for AD is greatly hindered by the low brain specificity and low permeability across BBB of the therapeutic agents. We developed an *in vitro* triple co-culture BBB model to characterize the brain targeting properties as well as the ability of designed liposomal gene carrier to translocate across BBB and deliver pDNA into brain cells. The BBB model was constructed with co-culture of glial and bEnd.3 cells and the integrity of the barrier was characterized by measuring TEER. Primary neuronal cells cultured in the bottom of well plate completed the *in vitro* triple co-culture BBB model which was used to assist the selection of suitable formulations for brain-targeted gene delivery, i.e., the carriers should be able to overcome this barrier layer and still be able to deliver the therapeutic gene into neuronal cells. The targeting efficiency of dual modified liposomes, Tf-Pen-liposomes, revealed the superior ability of the system to translocate across *in vitro* BBB and, thereafter, transfect primary neuronal cells as compared to liposomes without surface modification, or single-modified liposomes. These results suggested the contribution of dual modification to promote transport of liposomes across BBB and transfection in primary neuronal cells. Therefore, considering our *in vitro* findings, Tf-Pen-liposome might be able to overcome *in vivo* BBB and deliver pApoE2 to brain cells.

Our liposomal formulations were designed for intravenous administration. Therefore, it is important to determine the blood compatibility of the system prior *in vivo* administration. The low hemolytic activity of liposomal formulations at low phospholipid concentration were suggestive to be safe for intravenous administration (39). Based on previous studies published by our group, Tf-Pen-liposomes demonstrated superior brain targeting properties, greater *in vivo* BBB permeability and transfection of brain cells as compared to unmodified liposomes and single modified liposomes (either Tf or Pen) (21). Therefore we opted to evaluate *in vivo* liposome-mediated pApoE2 transfection using unmodified and Tf-Pen-modified liposomal formulations. The engineered brain-targeted liposomes showed *in vivo* brain targeting and gene delivery efficiencies. The *in vivo* results demonstrated that Tf-Pen-liposomes encasing chitosan-pApoE2 was capable of increasing significantly ($p <$ 0.05) ApoE levels in the brain of mice on single injection compared with unmodified liposomes encasing chitosan-pApoE2 complexes and equivalent dose of free plasmid ApoE2. Studies using AD mouse model have reported the potential clinical application of ApoE2 therapy for treatment of AD. Increasing ApoE2 levels in the brain of those animals decreased the endogenous Aβ as well as amyloid deposition which supported synaptic and neuritic plasticity (26,40,41). It is particularly encouraging to note that the current study demonstrated the effectiveness of designed brain-targeted liposomes-mediated ApoE2 gene therapy as a potential disease-modifying therapy for AD. Additional studies are needed to investigate the therapeutic potential of liposome-mediated ApoE2 gene transfer for treatment of AD using AD mouse model.

CONCLUSION

In this study, brain-targeted liposome for delivery of plasmid ApoE2 based on Tf receptor targeting with enhanced cellular penetration has been successfully developed as potential disease-modifying therapy for AD. The designed liposomal nanoparticles provided protection of plasmid ApoE2 against nuclease degradation. Liposome surface modified with Tf and Pen efficiently delivered pDNA and induced ApoE expression *in vitro*. Additionally, this system showed superior *in vitro* and *in vivo* BBB permeability. Tf-Pen-liposomes efficiently delivered the therapeutic gene into the brain of mice and increased ApoE expression, showing a better effect than the preparations without targeted-modifications. Therefore, the strategy for ApoE2 gene transfer across BBB using dual-modified liposomes for brain targeting and gene delivery has a great potential for prevention and treatment of AD. The therapeutic efficacy of the system will be further studied in AD mouse model.

ACKNOWLEDGMENTS AND DISCLOSURES

ABBREVIATIONS

AD	Alzheimer's disease
BBB	Blood brain barrier
CPP	Cell-penetrating peptide
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
LOAD	Late onset form of Alzheimer's disease
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
p/s	Penicillin and streptomycin
Pen	Penetratin
TEER	Transepithelial electrical resistance
Tf	Transferrin
TfR	Transferrin receptor

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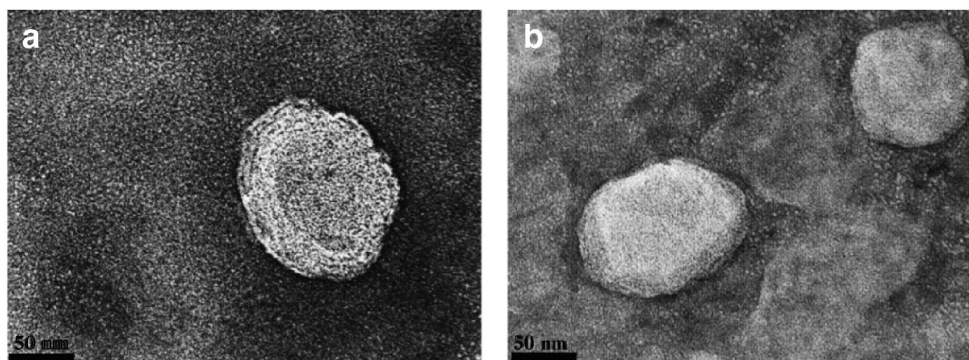
Fig. 1.
Transmission electron microscopy (TEM) images of Plain-liposomes (**a**) and PenTf-liposomes (**b**) which was negatively stained with 0.1% phosphotungstic acid aqueous solution (Scale 50 nm).
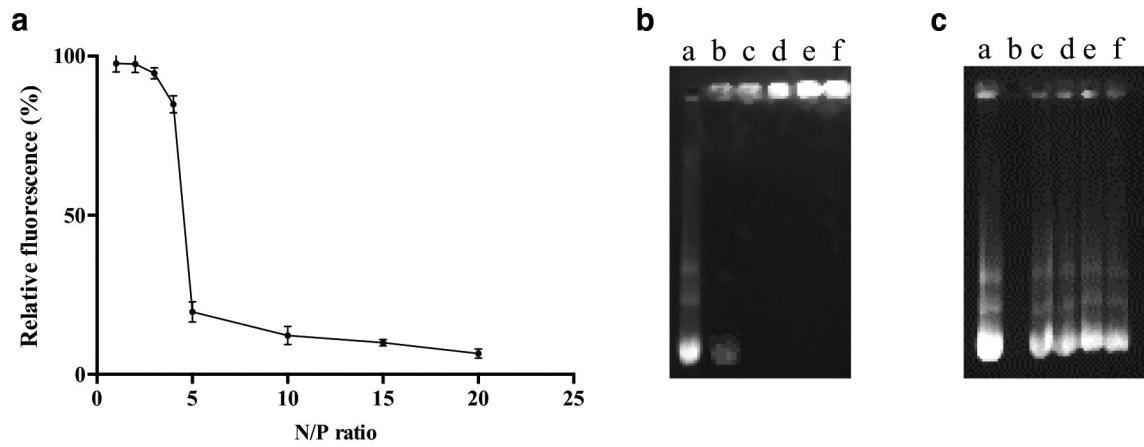
Fig. 2.
(**a**) Relative fluorescence of chitosan-pApoE2 complexes in different N/P ratios (1-5, 10, 15 and 20). Data are expressed as mean ± SD ($n = 4$). (**b**) Agarose gel electrophoresis of chitosan-pApoE2 complexes in different N/P ratios (1,5,10,15,20), lanes **b-f**, respectively). Naked pApoE2 (lane **a**) was used as control. (**c**) Protective effective of liposomal formulation containing chitosan-ApoE2 complexes (N/P 5) against nuclease degradation. Lane a, naked pApoE2; lane b, naked pApoE2 + DNase I; lanes c-f, Liposome, Tf-liposome, Pen-liposome, and Tf-Pen-liposomes containing chitosan-ApoE2 complexes, respectively + DNase I.
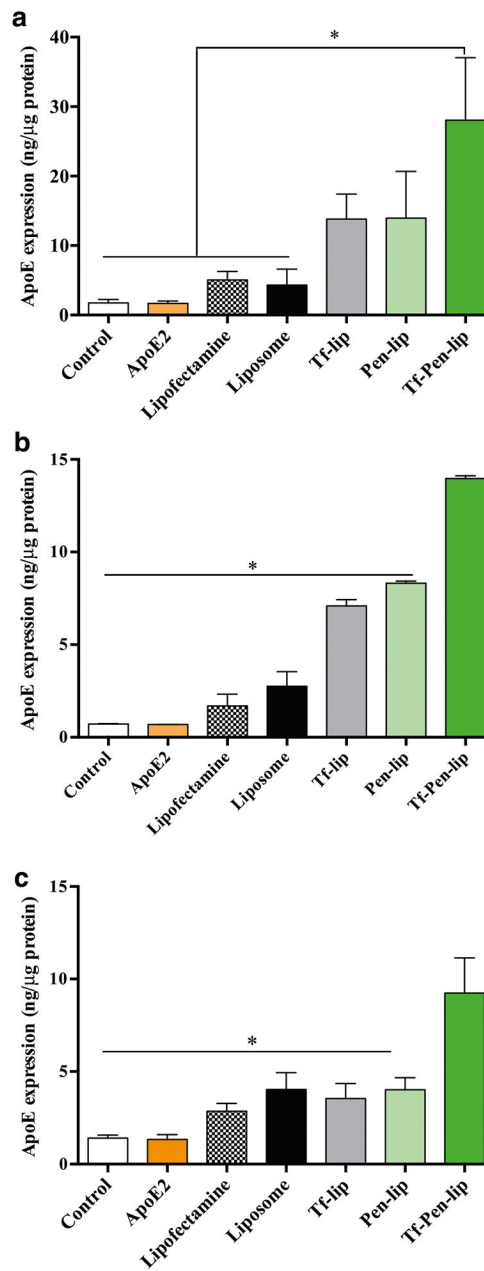
Fig. 3.
ApoE expression levels 48 h after transfection of bEnd.3 (**a**), glial (**b**) and primary neuronal cells (**c**) with Liposome, Tf-lip, Pen-lip and Tf-Pen-liposomes containing chitosan-pApoE2 (1 μg) complexes. Data are expressed as mean $\pm$ SD ($n = 4$). Statistically significant ($p < 0.05$) differences are shown as (*).
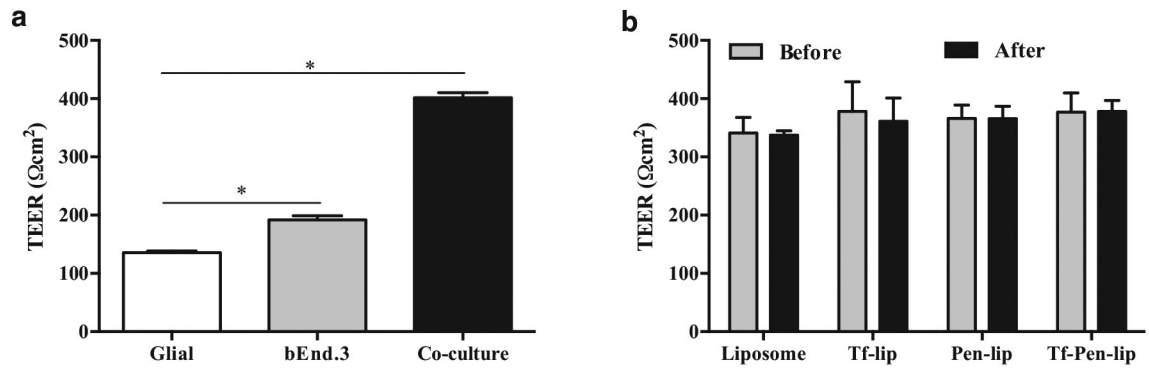
Fig. 4.
(**a**) Transendothelial electrical resistance (TEER, expressed as Ωcm^{-2}) of different *in vitro* BBB model constructed using glial and bEnd.3 monolayers and co-culture of bEnd.3 and glial. Significantly ($p < 0.05$) higher statistical difference in the TEER values between the groups was noted (*). (**b**) TEER of co-cultured *in vitro* BBB model before and after 8 h of transport study upon incubation with Liposome, Tf-lip, Pen-lip and Tf-Pen-liposomes. All data are expressed as mean ± SD ($n = 4$).

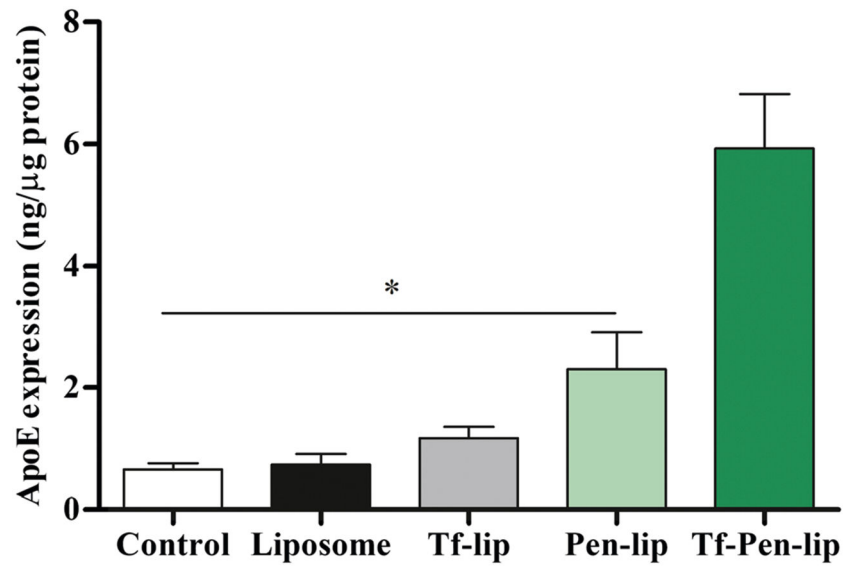**Fig. 5.**
Transfection efficiency of Liposome, Tf-lip, Pen-lip and Tf-Pen-liposomes containing chitosan-pApoE2 (1 μg) complexes in primary neuronal cells after transport through the *in vitro* BBB co-culture model as determined by ApoE ELISA kit. Data are expressed as mean ± SD ($n = 5$). Statistically significant ($p < 0.05$) differences are shown as (*).
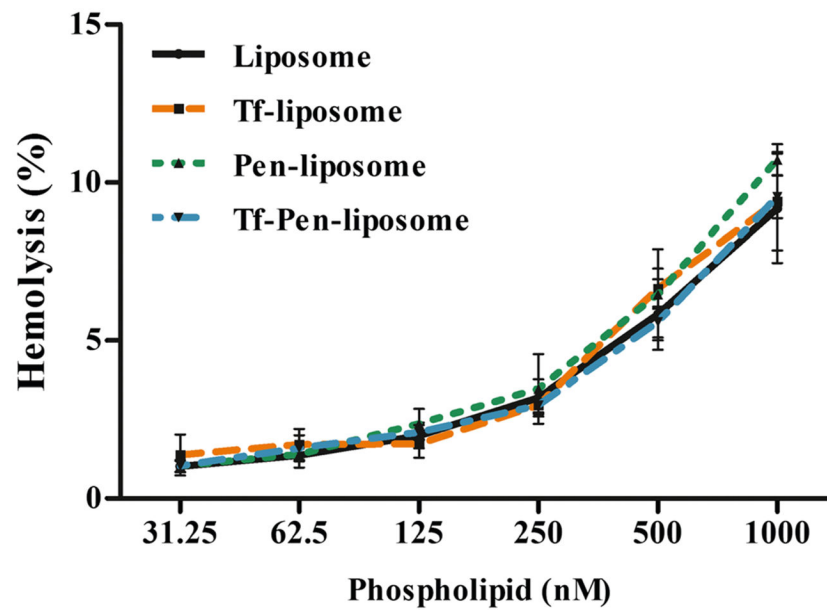
Fig. 6.
Hemolytic activity of Liposome, Tf-lip, Pen-lip and Tf-Pen-liposomes at different phospholipid concentrations (31.25–1000 μM). Hemolytic activity of 1% (v/v) Triton X-100 was considered as 100% hemolysis. Data are expressed as mean ± SD ($n = 4$).
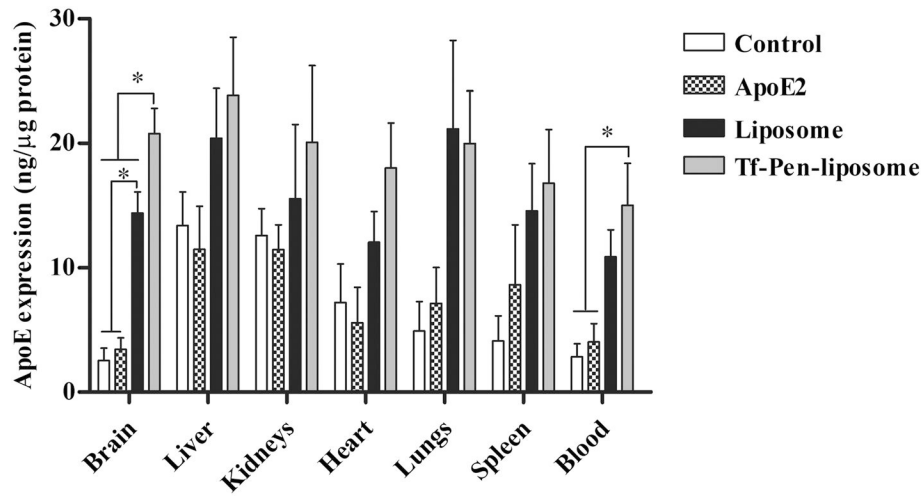
Fig. 7.
ApoE expression in C57BL/6 mice treated with liposomal formulations containing plasmid ApoE2 after 5 days of liposome administration. ApoE levels were quantified in different organs (brain, liver, kidneys, heart, lungs, spleen and blood) harvested from mice treated with plasmid ApoE2, Plain-liposome and Tf-Pen-liposomes containing chitosan-pApoE2 complexes (1 mg pDNA/kg body weight). Seven mice were used per group. Data are expressed as mean ± SD. Statistically significant differences ($p < 0.05$) are shown as (*).
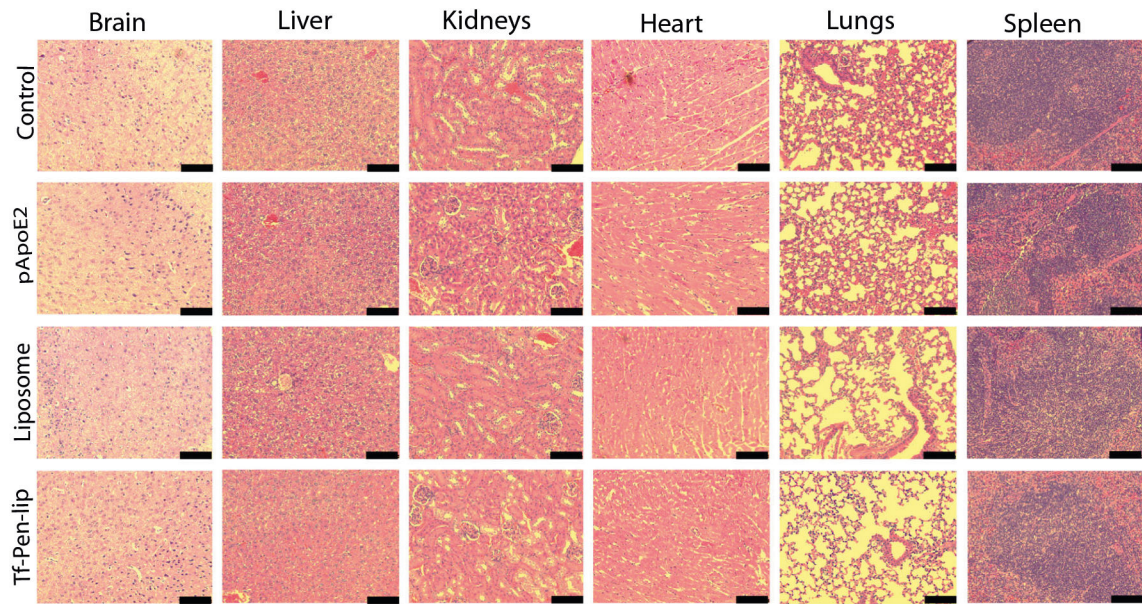
Fig. 8.
Representative tissue sections of mice stained with hematoxylin and eosin (H&E).
Mice were administered with saline solution (control), plasmid ApoE2, Liposome
encapsulating chitosan-pApoE2 complexes and Tf-Pen-liposome encapsulating chitosan-
pApoE2 complexes through tail vein injection. Scale bar, 100 μm.

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Table I

Liposome-pApoE2: Particle Size, PDI, Zeta Potential and EE

Liposomes	Particle size (nm)	PDI[a]	Zeta potential (mV)	EE[b]
Liposome	166.1 ± 1.57	0.24 ± 0.02	39.4 ± 3.22	92.8 ± 0.54%
Tf-liposome	156.8 ± 2.42	0.30 ± 0.03	17.3 ± 3.23	97.1 ± 1.20%
Pen-liposome	147.9 ± 4.34	0.23 ± 0.01	30.6 ± 1.29	96.5 ± 0.43%
Tf-Pen-liposome	144.9 ± 4.12	0.31 ± 0.04	23.0 ± 0.93	96.2 ± 1.04%

[a]PDI: Polydispersity index

[b]EE: Encapsulation efficiency