‘Sink effect’ of dually-decorated nanoliposomes on Aβ clearance in an in vitro model of the blood-brain barrier

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ABSTRACT

Plaques containing β-amyloid (Aβ42) peptides are one of the hallmarks of Alzheimer's disease (AD) and the reduction of Aβ is considered a primary therapeutic target (Mehta PD, Curr Alzh Res 2007). Studies in mouse models of AD have indicated that lowering Aβ42 levels in the brain can minimize the neurodegeneration (Boche D, Curr Opin Neurol 2005). Recently many strategies have been employed to reduce Aβ42 brain levels and one of them is based on the ‘sink effect’ hypothesis, that is the peripheral administration of Aβ42 binding agents able to reduce Aβ42 brain amount by sequestering it in the plasma.
This study for the first time investigates the potential effect of dually-decorated liposomes on the Aβ exchange across an in vitro model of the blood-brain barrier (BBB).
The results obtained show that liposomes significantly enhance the cellular uptake from the bottom side (corresponding in vivo to brain compartment) , and the passage to the apical side (corresponding in vivo to blood compartment) of Aβ42 across the BBB compared to Aβ42 alone.
Keywords: liposomes, Alzheimer, Abeta, sink effect, blood brain barrier

1 INTRODUCTION

Alzheimer Disease (AD) causes continuous deterioration of higher nervous functions, due to progressive and irreversible neurodegeneration of the limbic and association cortices, leading to the total loss of autonomy and eventually to death. Although substantial progress has been made in the scientific understanding of AD, there remains an urgent need to identify effective therapies and early detection strategies, in order to avert a financially overwhelming public health problem. Several evidences suggest that the overproduction and subsequent accumulation of β- amyloid (Aβ), a proteolytic fragment composed of 40 to 42 a.a. residues , derived from the membrane-associated amyloid precursor protein (APP), plays a central role in AD. This peptide is released from cells in a soluble form that progressively aggregates, forming oligomers, fibrils and ending with the deposition of extracellular plaques, detectable post-mortem in AD brains. Plaques are one of the morphological hallmarks of the disease, along with intracellular fibrillary tangles constituted of hyper-phosphorylated protein tau.). Recently many strategies have been employed to reduce Aβ brain levels and one of them is based on the ‘sink effect’ hypothesisthat brain and blood Aβ are in equilibrium through the blood brain barrier (BBB), and sequestration of Aβ in the blood by peripheral administration of Aβ binding agents may shift this equilibrium, drawing out the excess from the brain. This study for the first time investigates the potential effect of dually-decorated liposomes on the Aβ exchange across a Transwell in vitro model of the blood-brain barrier (BBB). In particular, we examined the effect of liposomes composed of sphingomyelin/ cholesterol/ phosphatidic acid and surface decorated with human ApoE-derived peptide, added to the apical side of the BBB model (corresponding in vivo to blood compartment), on Aβ42 withdrawal from the bottom compartment (corresponding in vivo to brain compartment) across the BBB.

2 MATERIALS AND METHODS

2.1 Preparation of liposomes

Liposomes were prepared by an extrusion method in 10 mM phosphate buffered saline buffer (PBS), pH 7.4 through a 100-nm pores polycarbonate filters and were composed of sphingomyelin (Sm) and cholesterol (Chol) at 1:1 molar ratio. Sm and Ch were mixed with 5% molar phosphatidic acid (PA). Liposomes contained also 2.5 molar% of mal-PEG-PE for subseguent functionalization. Briefly, lipids were mixed in chloroform/methanol (2:1, v/v) and dried under a gentle stream of nitrogen followed by a vacuum pump for 3 h to remove traces of organic solvent. The resulting lipid film was rehydrated in phosphate-buffered saline (PBS), vortexed and then extruded 10 times at 55 °C through a stack of two polycarbonate filters (100-nm pore size diameter) under 20 bar nitrogen pressure with an extruder. liposomes were separated from possible unincorporated material by size-exclusion chromatography using PD-10 column and PBS as the eluent. Lipid recovery after PD-10 column was assessed by assaying the individual components or by measuring the radioactivity associated. Phospholipid recovery after extrusion was determined by phosphorous assay using the method of Stewart.
Two different radioactive lipid tracers (^{3}H-Sm and ^{14}C-PA) were also present for quantification of cellular uptake, permeability measurements across the blood brain barrier and binding experiments.
Lipid recovery after extrusion was evaluated by assaying the individual components: size, polydispersity index and zeta potential were obtained using a ZetaPlus particle sizer

and zeta-potential analyzer (Brookhaven Instruments, Holtsville, New York) at 25°C in PBS by dynamic light scattering with a 652-nm laser beam. size and polydispersity were obtained from the intensity autocorrelation function of the light scattered at a fixed angle of 90 degrees. The correlation function was analyzed by means of a two-cumulant expansion. Each measurement was performed under an electrical field of 29.7 V/cm.22 Standard deviations were calculated from at least three measurements. Stability was measured in buffer byfollowing size and polydispersity index by dynamic light scattering for 3 days.

2.2 Functionalization of liposomes with ApoE peptides

The sequence corresponding to a.a. residues 141–150 of human ApoE peptide and its tandem dimer repeat $(141–150)_2$ were synthesized on an automated Applied Biosystems synthesizer model 433A (Applied Biosystems, Foster City, California) at 0.1 mM scale with NOVASYN-TGA resin, using Fmoc-protected L-amino acids.. The peptides were bearing at the C-terminal a tryptophan residue for fluorescence monitoring and ended with cysteine residue for covalent coupling with mal-PEG-PE in the liposome bilayer. Amino acids were activated by reaction with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetra fluoroborate and N,N-diisopropylethylamine. A capping step with acetic anhydride after the last coupling cycle of each amino acid was included. Peptides were cleaved from the resin with trifluoroacetic acid/water/3,6-dioxa-1,8-octanedithiol (90:5:5 vol/vol/vol, 180 min at 25°C), precipitated, and washed with diethyl ether. Crude peptide was then purified by reverse-phase high-performance liquid chromatography on a semipreparative C18 column (Symmetry 300; Waters Corporation, Milford, Massachusetts), and peaks collected were characterized by matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF).
mApoE or dApoE or tat-peptide was added to liposomes in PBS to give a final peptide:mal-PEG-PE molar ratio of 1.2:1, and incubated over night at room temperature to form a thioether bond with mal-PEG-PE. Liposomes decorated with the peptide (ApoE-liposomes) were separated from unbound peptide using PD-10 column . The yield of coupling of the peptide to liposomes was assessed by measuring the tryptophan fluorescence intensity (λ_{ex} = 280 nm) of the incubation mixture and of ApoE-liposomes recovered from the PD-10 column. Spectra were recorded between 300 and 450 nm using a Cary Eclipse spectrofluorometer (Varian). The amount of peptide bound to liposomes was calculated from the fluorescence intensity of a known amount of the peptide dissolved in PBS, taken as the standard.

2.3 Exchanges of Aβ across an in vitro model of BBB

For transport experiments across a cell monolayer, Human brain endothelial cells (hCMEC/D3) were obtained from Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France). hCMEC/D3 cells cultured between passage 25 and 35 were used. For culturing, the cells were seeded at a concentration of 27000 cells/cm^2 and grown in tissue culture flasks coated with 0.1mg/ml rat tail collagen type 1. Under these culture conditions, hCMEC/D3 cells express P-Gp polarised on the apical membrane.
A cell monolayer was usually formed 3 days after seeding, as judged by three criteria: (1) the cells formed a confluent monolayer without visible spaces between cells under a light microscope; (2) the height of the culture medium in the upper chamber had to be at least 2 mm higher than that in the lower chamber for at least 24 hours; and (3) a constant transendothelial electrical resistance (TEER) value, measured using an EVOM Endohmchamber (World Precision Instruments, Sarasota, Florida) was obtained (typically between days 6 and 8). Wells were used when TEER value was higher than 100 Ω×cm2.
All the permeability experiments were performed in serum-free medium at 37°C.
In parallel experiments, the efflux of the hydrophilic marker [14C]sucrose (200 μM) added in the upper chamber was measured to evaluate the paracellular permeability. At the end of the experiments, TEER and [14C]sucrose PEs were again determined so as to confirm that sample application had resulted in no adverse effects on tight junction function.
Liposomes were prepared adding radiolabelled lipids, [^{3}H]-sphingomyelin and [^{14}C]-phosphatidic acid, as tracers [4].
Aβ 42 was added into the lower chamber at 300 nM concentration. Liposomes were added into the upper chamber at 100 or 200 nm total lipid concentation.
The clearance of the Aβ 42 from the basolateral to the apical side of the BBB model , and the uptake of the peptide was followed by ELISA assay, whereas the LIPOSOMES distribution was followed by radioactivity counting

3 RESULTS

The functionalization of liposomes with PA confers to them high binding affinity for Aβ with Kd 60 nM as assessed by SPR. These NP proved their potential to be used for the therapy of AD by preventing, in vitro, neurotoxicity of Aβ peptide, and Aβ-induced Tau phosphorylation [1-3].
Liposomes were characterized by DLS. Sm/Chol liposomes containing 5% PA displayed a mean diameter of 114 nm, a PDI of 0.318 and a Z potential −28.38±0.059 mV. After coupling with ApoE peptide, the size of liposomes slightly increased to 136 nm with PDI 0.198 and z potential −27.74±0.018. The high negative zeta

potential and the low PDI of ApoE-liposomes indicate that the liposomes are stable after functionalization with ApoE peptide, in according with previous data [5,6]. Double functionalization (both for binding Abeta and for crossing the BBB) enhances NP cell uptake and transport across the BBB model without affecting the affinity for Abeta that remained in the same order of magnitude (data not shown).
The fluxes of Abeta across the Transwell model of BBB (Fig.1) when the TEER was about 100 Ωxcm2 (fig.2) were followed by adding Aβ42 in the lower compartment, then adding liposomes in the upper compartment (corresponding in vivo to blood compartment) and assaying i) Aβ42 in the upper and lower chamber and ii) Aβ42 uptaken by the cells after a fixed time (120 min).

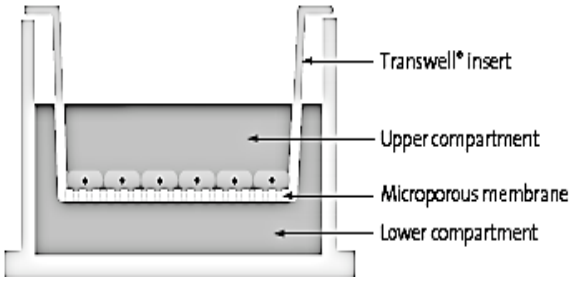

Figure 1- Illustration of the transwell system utilized as a model of blood-brain barrier. hCMEC/D3 cells were grown as a monolayer over a microporous membrane filter. The fluxes across the barrier were followed by assaying the components in the upper and lower chamber after 120 min incubation.

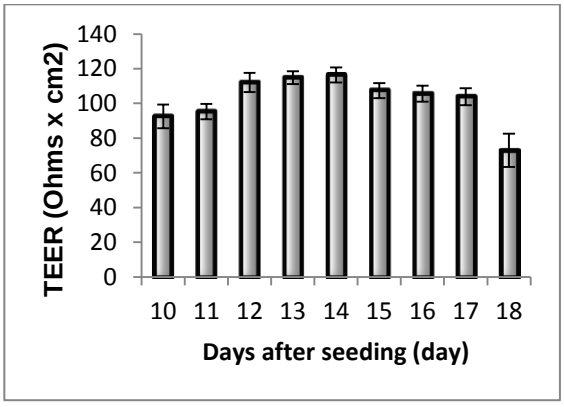

Fig.2- TEER of the monolayer hCMEC/D3 cell culture in the Transwell system, measured at different times of culture

The results showed that liposomes and Aβ42 treatment did not affect the BBB functional and bioelectrical properties (fig. 3).

We observed that these liposomes significantly enhanced the cellular uptake of Aβ42 from the bottom side compared to the amount of Aβ42 uptaken from the cells in the absence of liposomes (fig. 3) . Moreover, the liposomes treatment strongly enhanced (+110%) the passage of Aβ42 from the bottom side (corresponding in vivo to brain compartment) to the upper side (corresponding in vivo to blood compartment) compared to Aβ42 alone. Moreover, the rate of liposomes-mediated Aβ42 clearance was time- and lipid dose-dependent (Fig.3).

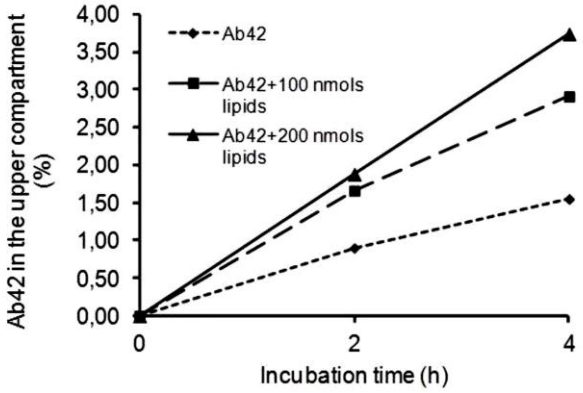

Fig. 3 . Ab42 transcytosis across the hCMEC/D3 cell monolayer alone or in presence of different amount of liposomes.

4 DISCUSSION

This study provide rationale for the use of Aβ binding liposomes as a treatment strategy in AD and suggests that this particular liposomes formulation is important in the brain-to-blood transport of Aβ 42. In general, nanoparticles that sequester plasma Aβ could reduce or prevent brain amyloidosis, which would enable the development of new therapeutic agents for AD treatment

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