The hunt for brain Aβ oligomers by peripherally circulating multi-functional nanoparticles: Potential therapeutic approach for Alzheimer disease

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Abstract

We previously showed the ability of liposomes bi-functionalized with phosphatidic acid and an ApoE-derived peptide (mApoE-PA-LIP) to reduce brain Aβ in transgenic Alzheimer mice. Herein we investigated the efficacy of mApoE-PA-LIP to withdraw Aβ peptide in different aggregation forms from the brain, using a transwell cellular model of the blood–brain barrier and APP/PS1 mice. The spontaneous efflux of Aβ oligomers (Aβo), but not of Aβ fibrils, from the 'brain' side of the transwell was strongly enhanced (5-fold) in presence of mApoE-PA-LIP in the 'blood' compartment. This effect is due to a withdrawal of Aβo exerted by peripheral mApoE-PA-LIP by sink effect, because, when present in the brain side, they did not act as Aβo carrier and limit the oligomer efflux. *In vivo* peripheral administration of mApoE-PA-LIP significantly increased the plasma Aβ level, suggesting that Aβ-binding particles exploiting the sink effect can be used as a therapeutic strategy for Alzheimer disease.

From the Clinical Editor

Alzheimer disease (AD) at present is an incurable disease, which is thought to be caused by an accumulation of amyloid-β (Aβ) peptides in the brain. Many strategies in combating this disease have been focused on either the prevention or dissolving these peptides. In this article, the authors showed the ability of liposomes bi-functionalized with phosphatidic acid and with an ApoE-derived peptide to withdraw amyloid peptides from the brain. The data would help the future design of more novel treatment for Alzheimer disease.

Alzheimer disease (AD) is the most common form of dementia in the elderly, with no current therapy or definite diagnosis. Brain accumulation of amyloid-β (Aβ) peptides, eventually deposited as plaques, is one of the pathological hallmarks of AD.[1] Aβ accumulation has been hypothesized to result from an imbalance between Aβ production and clearance; indeed, Aβ clearance seems to be impaired in both early and late forms of AD and may contribute to the onset and progression of the disease.[2] Moreover, additional evidence suggests that about 80-90% of AD patients show cerebral amyloid angiopathy (CAA), characterized by Aβ accumulation in brain blood vessel walls,[3] altering the functionality of the BBB.[4] Among the different aggregation forms of Aβ, a body of evidence indicates that soluble Aβ oligomers (Aβo), rather than insoluble deposits, such as Aβ fibrils (Aβf) and plaques, are primarily responsible for both neurodegeneration and synaptic impairment in AD. Therefore, in the last years, many efforts have focused at preventing Aβo formation or disassembling existing aggregates.[5, 6, 7] Recently, a therapeutic strategy based on lowering the levels of soluble Aβ assemblies in the brain and in the cerebral blood vessel exploiting the peripheral-sink effect has been proposed.[8] To this purpose, it has been postulated that brain and plasma Aβ pools are in equilibrium through the BBB, and that the peripheral sequestration of Aβ may shift this equilibrium toward the peripheral blood circulation, eventually drawing out the

excess from the brain and/or from the brain vessels.[8] DeMattos et al reported that peripheral administration of a monoclonal anti-Aβ antibody to transgenic AD mice models resulted in an increase of Aβ in the plasma, despite minimal entry of the antibody into the brain, suggesting the equilibrium of Aβ between the brain and plasma.[9], [10] Because there are several problems associated with immunotherapy of AD,[11] the use of molecules that are unrelated to antibodies, such as gelsolin, GM1 ganglioside[8] or an extract from the root of *Withania somnifera*, has been proposed.[12] The peripheral administration of these compounds in AD animal models was suggested to reduce the level of Aβ in the brain because of the sink effect. Therefore, it is conceivable that the design of compounds that bind Aβ with high affinity could reduce or prevent brain Aβ deposition in AD patients. In this context, nanoparticles are considered a promising tool due to the possibility of multi-functionalization of their surface with several copies of Aβ-specific ligands, increasing the affinity for Aβ by multivalent interactions.[13] This feature makes them good candidates as sink effect promoters. We have previously described liposomes (LIP) embedding acidic phospholipids (in particular phosphatidic acid, PA) and surface-decorated with a modified human ApoE-derived peptide (mApoE), i.e. mApoE-PA-LIP, which have very high binding affinity to Aβ[6] In the present study, we investigated the efficacy of mApoE-PA-LIP to withdraw Aβ peptide in different aggregation forms from the brain, using a transwell cellular model of the blood-brain barrier and APP/PS1 mice.

Methods

Materials

All chemical reagents were from Sigma–Aldrich, Milano, Italy. Bovine brain sphingomyelin (Sm), cholesterol (Chol) and 1,2-stearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(poly(ethylene glycol)-2000)] (mal-PEG-PE) were purchased from Avanti Polar Lipids (USA). Dimyristoyl phosphatidic acid (PA), Aβ$_{1-42}$ peptide and 1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma–Aldrich, Milano, Italy. [^{3}H]-propranolol and [^{3}H]-Sm were purchased from PerkinElmer. FITC-dextran and bovine serum albumin (BSA) were from Sigma Aldrich, Milano, Italy. Polycarbonate filters for extrusion procedure were purchased from Millipore Corp., Bedford, MA. Extruder was from Lipex Biomembranes, Vancouver, Canada. All the media and supplements for cell cultures were supplied by Invitrogen Srl, Milano, Italy. The hCMEC/D3 cell line was obtained under license from Institut National de la Sante et de la Recherche Medicale (INSERM, Paris, France). Rat type I collagen, 1/100 chemically defined lipid concentrated, and all the media and supplements for cell cultures were from Invitrogen Srl (Milano). For determination of Aβ$_{1-42}$, human β amyloid (1-42) ELISA kits (IBL) were used. Low-range rainbow molecular weight marker, horseradish peroxidase (HRP)-conjugated goat IgG anti-mouse antibody and ECL reagents were purchased from Amersham Biosciences (Castle Hill, NSW, Australia). Mouse monoclonal Aβ$_{1-42}$ antibody mAβ 6E10 was from Signet (Dedham, MA).

Preparation and characterization of liposomes

mApoE-PA-LIP composed of a Sm/Chol matrix (1:1, M:M) and bi-functionalized with PA (5 mol%) and with the peptide NH2-CWG-LRKLRKRLLR-CONH2 (MW 1698.18 g/mol, mApoE, 1.25 mol%) derived from the receptor-binding domain (a.a. residues 141-150) of human ApoE, modified by adding the tri-peptide CWG at the N-term, were prepared as previously described[6] by extrusion procedure using 100 nm diameter filter pores (Millipore). To prepare larger mApoE-PA-LIP, 400 nm diameter filter pores were used. Non-functionalized LIP (control LIP, composed by Sm/Chol 1:1), LIP mono-functionalized with PA (PA-LIP) or mono-functionalized with mApoE (mApoE-LIP), were used as a control. In some instance, [^{3}H]-sphingomyelin (less than 0.0001% of

total lipids) was added as a tracer. Size and polydispersity index (PDI) of diluted LIP dispersions (at a final lipid concentration of 0.5 mg/ml in PBS pH 7.40) were measured by dynamic light scattering (DLS) technique (Brookhaven Instruments Corporation, Holtsville, NY, USA). ζ-Potential was measured for the same samples by using an interferometic Doppler velocimetry with the same instrument equipped with ZetaPALS device. Stability was measured in PBS by following size and PDI by DLS for 5 days.[6]

Aβ peptide preparation and characterization

Aβ$_{1-42}$ peptide was solubilized in HFIP at 1 mg/ml concentration. The peptide was allowed to air dry in a chemical fume hood overnight. The resulting clear film was stored at − 20 °C. The peptide was resuspended before use in DMSO at a concentration of 5 mM and bath sonicated for 10 min to achieve a monomer-enriched preparation (Aβm). This preparation was diluted to 100 μM in cell culture medium and incubated 24 h at 4 °C to obtain an oligomer-enriched preparation (Aβo). Alternatively, the 5 mM Aβ preparation was diluted to 100 μM in 10 mM HCl and incubated at 37 °C for 24 h in order to obtain a fibril-enriched preparation (Aβf).[14] The aggregation state of Aβm and Aβo (not Aβf because too large to be resolved) was assessed by SDS-PAGE gel electrophoresis on a 4-20% Tris-Glycine gel (Thermo Scientific, Milano), followed by immunoblotting analysis using 6E10 anti-Aβ$_{1-42}$ antibody (1:1000 dilution, Covance, Italy). Aβ assemblies were visualized with enhanced chemiluminescence (ECL) by ImageQuant LAS4000.[6] The morphology of Aβo and Aβf (not Aβm because below the AFM resolution limit) was assessed by Atomic Force Microscopy as previously described.[15]

Binding of mApoE-PA-LIP to Aβo or Aβf investigated by ultracentrifugation density gradient

The binding of mApoE-PA-LIP to Aβo or Aβf was investigated as previously described.[13] Briefly, Aβ samples were incubated with radiolabeled mApoE-PA-LIP (1:2.5, peptide:lipids) in PBS pH 7.4 at 37 °C from 15 min. After incubation the peptide bound to LIP was separated from free peptide by flotation in a discontinuous sucrose density gradient performed as described.[13] After ultracentrifugation in a Beckman MLS 50 rotor at 140.000 × g for 2 h in polycarbonate tubes, 10 fractions were collected from the top of the gradient and assayed for lipid and peptide content, by measuring the lipid-associated radioactivity by liquid scintillation and by dot-blot procedure on a PVDF membrane, respectively. The proportion of Aβ bound to LIP was expressed as the % ratio between the amount of peptide in the fractions 1-5 over the total peptide amount, digitally semi-quantitatively estimating the chemiluminescent spots on PVDF membrane using ImageQuant LAS4000 software.

In vitro model of BBB

Human brain endothelial cells (hCMEC/D3) were obtained from Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France) and used as a representative human BBB model. hCMEC/D3 cells were cultured at 37 °C, 5% CO_2/saturated humidity in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS), 1% penicillin–streptomycin, 1.4 μM hydrocortisone, 5 μg/mL ascorbic acid, 1/100 chemically defined lipid concentrate (Invitrogen), 10 mM HEPES and 1 ng/mL basic FGF (bFGF).[6, 16] To set up the BBB model, hCMEC/D3 cells, between passage 25 and 35, were seeded at a concentration of 60,000 cells/cm^2 onto collagen-coated (4 μg/cm^2 rat tail collagen type 1 Invitrogen) transwell filters (polycarbonate 12-well, pore size 0.4 μm, translucent membrane insert 1.12 cm^2; Euroclone) to establish a polarized monolayer.[6, 17, 18] The layer of endothelial cells separates this system into an apical ('blood' side; 0.5 ml of volume) and basolateral ('brain' side; 1 ml of volume) compartment.

Impact of Aβ on cell monolayer properties

hCMEC/D3 cells were cultured on a transwell system as described above. Bioelectrical, morphological and functional properties of the cell monolayers were checked both in the presence or absence of 500 nM Aβ in the basolateral compartment. Bioelectrical properties of cell monolayer were checked by measuring the transendothelial electrical resistance (TEER) with STX2 electrode Epithelial Volt-Ohm meter (World Precision Instruments, Sarasota, Florida). Morphological and functional properties of cell monolayer were evaluated by examining cells under microscope, by measuring the paracellular permeability of 250 μM FITC-dextran (MW 4 kDa) and the transcellular permeability of 76 nM [^{3}H]-propranolol (0.5 μCi), as previously described.[6] Cell viability was also assessed by MTT assay.[19]

Aβ cellular uptake and exchange across the BBB model in presence of liposomes

For Aβ exchange across the BBB model studies, hCMEC/D3 were cultured on a transwell system as described above. 500 nM Aβ was added to the basolateral compartment, exposed to the complete medium and different doses (0-200 nmol total lipids) of LIP (alone or in presence of different amounts of Aβo) were added to the apical compartment, exposed to PBS (Figure 1). In some instances, in order to mimic *in vivo*-like conditions, PBS-BSA 4% (w/v) solution was added to the apical compartment and PBS-BSA 0.015% (w/v) solution was added to the basolateral compartment. After different times of incubation (up to 4 h), aliquots were collected from the apical and basolateral compartment and the Aβ content was measured by ELISA assay (IBL 96-well), following the manufacturer's instructions, and absorbance was read at 450 nm using a spectrophotometer Victor (Victor2 PerkinElmer). The concentration of Aβ was determined according to the standard curve prepared, as suggested by ELISA kit protocol. The Aβ endothelial permeability (EP) across the cell monolayer was calculated as described[20]:
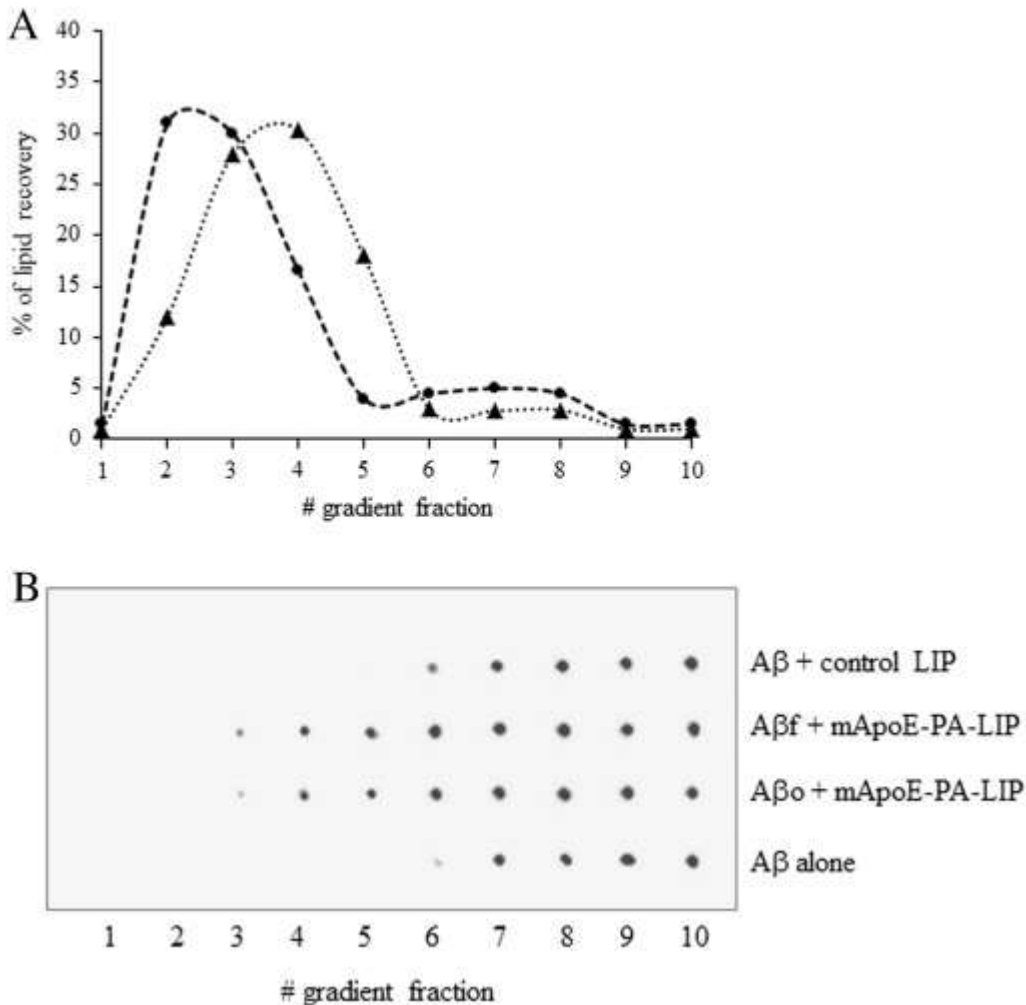
Figure 1. Binding of liposomes to Aβ by ultracentrifugation assay.

Mixtures of Aβ$_{1-42}$ (oligomers- or fibrils-enriched samples) with liposomes (containing tritiated sphingomyelin as a tracer) were purified by ultracentrifugation on a discontinuous sucrose density gradient and 10 fractions were collected from the top of the gradient. **(A)** Representative distribution of the liposome-associated radioactivity in the gradient fractions, before (dotted line) and after binding with Aβ (punctuated line). **(B)** Representative distribution of Aβ in the different fractions of the gradient detected by dot-blot assay using anti-Aβ$_{1-42}$ 6E10, followed by ECL detection. The spot intensity associated with each fraction was determined using the LAS4000 software. 1-5 fractions represent the Aβ bound to liposomes; 6-10 fractions represent the free peptide.

where:

X = amount of drug (Aβ in our case) in the receptor chamber (upper compartment in our case) = nmol

Cd = Aβ concentration in the donor chamber (bottom compartment in our case) = nmol/cm^3

Δt = time = min

Surface area = area of transwell inset filter = cm^2

Cells were collected with 200 μl of lysis buffer 2% Triton X-100 with the addition of protease inhibitor cocktail (Sigma) and an aliquot was used to measure the Aβ content by ELISA assay to determine the Aβ cellular uptake.

Liposomes exchange across the BBB model

For LIP exchange across the BBB model, hCMEC/D3 were cultured on transwell system as described above. mApoE-PA-LIP (200 nmol total lipids), containing [^{3}H]-Sm (0.001 mol%) as a tracer, were added to the basolateral compartment in a medium containing 500 nM Aβ and incubated at 37 °C for 2 h. The EP of LIP across the cell monolayers was estimated by measuring the radioactivity at different times (up to 2 h) of [^{3}H]-Sm in the apical and basolateral compartment by liquid scintillation counting, and calculated as described.[20]

Animal treatment

The double transgenic mice (APP/PS1) used in the present study incorporate a human APP construct bearing the Swedish double mutation and the exon-9-deleted PSEN1 mutation: B6.Cg-Tg (APPSwe, PSEN1dE9) 85Dbo/J (Jackson Laboratory, Bar Harbor: stock no. 005864). The genotype of the mice was confirmed by PCR of DNA isolated from tail biopsies.[21] All animal care and handling strictly followed the current Italian legislation and guidelines, and those of the European Commission (directive 2010/63/EU).

APP/PS1 mice (n = 7 mice/group) were intraperitoneally (I.P.) injected once every other day with mApoE-PA-LIP (100 μl, 73.5 mg of total lipids/kg) or with PBS as a control (100 μl), as already described,[22] with changes concerning the age of animals (16 months of age) and the duration of the treatment (two weeks). At the end of treatment, animals were sacrificed and blood was collected by cardiac puncture and processed for plasma separation. Brains were dissected, weighed and processed to extract Aβ[22] Aβ levels in plasma and brain were quantified by ELISA assay, as described.[22], [23]

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed by Student's *t* test. A *P*-value < 0.05 was considered statistically significant.

Results

Characterization of liposomes

In the present study, we utilized previously described mono- and bi-functionalized liposomes: PA-LIP, mApoE-LIP and mApoE-PA-LIP.[6], [16], [24] Non-functionalized LIP have been used as a control. Size, polydisperisity and ζ-potential values are reported in Table 1. DLS analysis showed that LIP were monodispersed and their size remained constant, within the experimental error, for up to 5 days (data not shown). The yield of LIP surface functionalization with mApoE peptide ranged between 50% and 60%, according to data already published.[16], [24]

Table 1. Physicochemical features of liposomes.

Liposomes	Diameter (nm ± SD)	PDI	ζ-Potential (mV ± SD)
Control LIP	108.1 ± 2	0.099	− 25.51 ± 0.82

Liposomes	Diameter (nm ± SD)	PDI	ζ-Potential (mV ± SD)
PA-LIP	114.7 ± 5	0.109	− 48.22 ± 2.34
mApoE-LIP	119.3 ± 7	0.123	− 16.41 ± 1.36
mApoE-PA-LIP	139.4 ± 9	0.143	− 18.32 ± 2.09

Control LIP, control liposomes composed of Sm/Chol 1:1 M/M. PA-LIP, liposomes functionalized with 5 mol% of phosphatidic acid. mApoE-LIP, liposomes functionalized with 1.25 mol% of mApoE peptide. mApoE-PA-LIP, liposomes bi-functionalized with 5 mol% of phosphatidic acid and 1.25 mol% of mApoE peptide. PDI, polydispersity index. SD, standard deviation.

Characterization of Aβ samples

Aβ samples were prepared as described above and the aggregation state was assessed by SDS-PAGE/WB and atomic force microscopy (AFM). The results showed that Aβo samples were enriched in small assemblies (MW < 20 kDa) (Figure S1, *A*, lane b; Figure S1, *B*) as compared to monomer-enriched ones (Figure S1, *A*, lane a) used as a control. AFM observations indicated that Aβo (Figure S1, *B*) had a spherical morphology and that no fibrils were present in the samples. Size distribution in AFM images appears to be coherent with SDS-PAGE/WB results. Aβf (Figure S1, *C*), as already reported in literature,[25] appeared unbranched, bowed, and several micrometers long, with an apparent height of 6 nm.

Binding of mApoE-PA-LIP to Aβ

The ability of mApoE-PA-LIP to bind Aβo or Aβf was assessed by ultracentrifugation on a discontinuous sucrose density gradient allowing to separate Aβ bound to LIP from the free peptide. Ten fractions were collected from the gradient and analyzed for lipid and peptide content by radiochemical technique and dot-blot assay, respectively. The results (Figure 1) showed that mApoE-PA-LIP were able to bind both Aβo (15.5% of Aβo bound to LIP) and Aβf (23.7% of Aβf bound to LIP), as compared to non-functionalized ones (control LIP). The binding to Aβ induced a change in liposomes density modifying the lipid distribution along the gradient fractions, as already reported.[26]

In vitro BBB transwell model

To investigate mApoE-PA-LIP capacity to hunt Aβo by peripheral-sink effect, we set-up an *in vitro* BBB transwell model (Figure 2) composed by a monolayer of polarized endothelial cells seeded on a porous membrane, allowing an apical compartment ('blood' side) physically separated from a basolateral one ('brain' side). Immortalized human brain capillary endothelial cells (hCMEC/D3) were used as representative of human BBB model and the transwell system was characterized by measuring bioelectrical, morphological and functional properties. Trans-electrical endothelial resistance (TEER) was monitored during cell monolayer's formation and was found to gradually increase from $125.19 \pm 5.6 \ \Omega\cdot cm^2$ (at 12 days *in vitro*, DIV) to $149.90 \pm 7.9 \ \Omega\cdot cm^2$ (at 15 DIV). At 15 DIV, endothelial permeability (EP) values for [³H]-propranolol and FITC-dextran (MW 4000 Da) were in the order of $2.3 \pm 0.06 \times 10^{-3}$ cm/min and $1.02 \pm 0.04 \times 10^{-6}$ cm/min, respectively, in agreement with the values reported in literature.[27] At 15 DIV, 500 nM Aβo were added to the basolateral compartment of the transwell system and the impact of Aβo on cell monolayer properties was checked. The results showed that the basolateral exposure of cell monolayer to Aβo did not significantly affect TEER values ($145.72 \pm 4.35 \ \Omega\cdot cm^2$ after 2 h of incubation; $142.80 \pm 1.93 \ \Omega\cdot cm^2$ after 4 h of incubation) nor EP of trans- and para-cellular probes (EP [³H]-propranolol = $2.1 \pm 0.05 \times 10^{-3}$ cm/min; EP FITC-dextran = $1.55 \pm 0.03 \times 10^{-6}$ cm/min).

Moreover, the treatment did not affect cells viability (> 95% cells viability respect to untreated cells), as assessed by MTT assay. Similar results were also obtained with the addition of 500 nM Aβf in the basolateral compartment (data not shown).

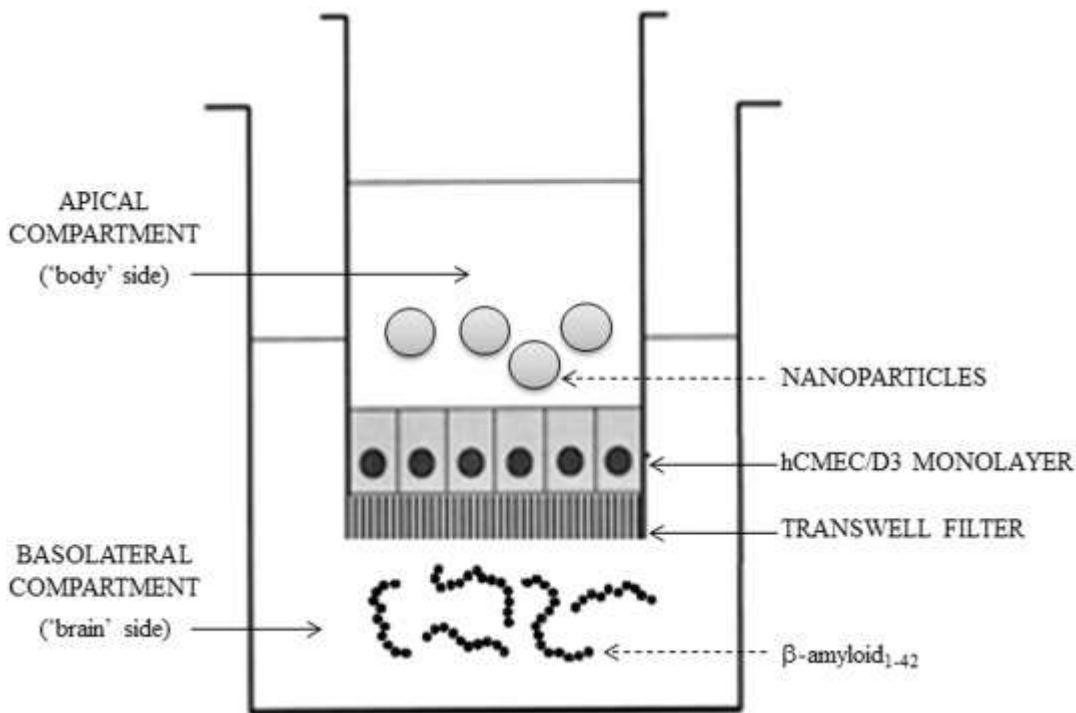Figure 2. Graphical representation of the transwell system.

Aβ clearance across the in vitro BBB model by mApoE-PA-LIP

Aβ passage across the BBB model (from the basolateral to the apical transwell compartment) was measured by ELISA assay in the presence or absence of LIP in the apical compartment.

The results showed that the amount of Aβo measured in the apical compartment increased over time (Figure 3), with an EP value of $2.62 \pm 0.3 \times 10^{-5}$ cm/min, in accordance with the literature.[28] On the contrary, Aβf efflux from the basolateral compartment was not detectable by ELISA assay.
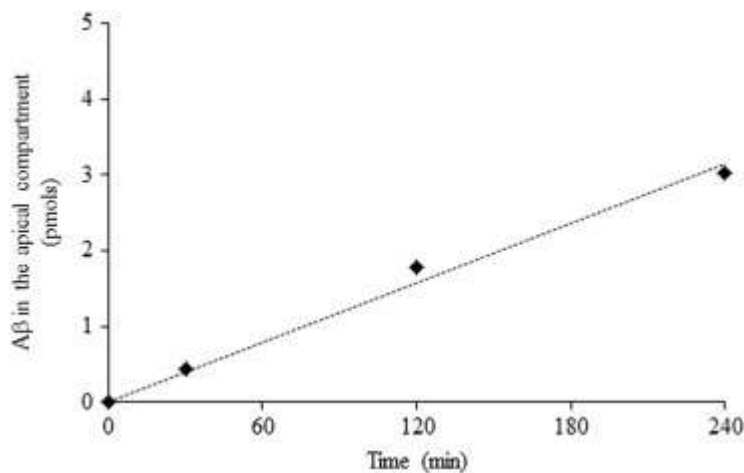

Figure 3. Aβ exchange across the BBB model.

hCMEC/D3 cells were cultured on transwell system and 500 nM oligomers-enriched Aβ sample was added to the medium in the basolateral compartment and the amount of peptide in the apical compartment was measured by ELISA assay after different incubation times (0-240 min). The amount (pmol) of Aβ recovered in the apical compartment in the function of time is shown.

When different functionalized LIP (200 nmol total lipids) were present in the apical compartment, the basolateral-to-apical Aβo EP increased, with respect to the EP of Aβo alone (Figure 4, A). In particular, the highest EP value (5-fold increase respect to EP of Aβo alone) was registered when mApoE-PA-LIP were present in the apical compartment. Additionally, the basolateral-to-apical EP of Aβo was mApoE-PA-LIP dose-dependent, increasing with the amount of LIP in the apical compartment (Figure 4, B). On the contrary, when the experiments were carried out with Aβf, after 2 h of incubation with mApoE-PA-LIP (200 nmol total lipids) in the apical compartment, no peptide was detectable by ELISA assay in the same compartment.
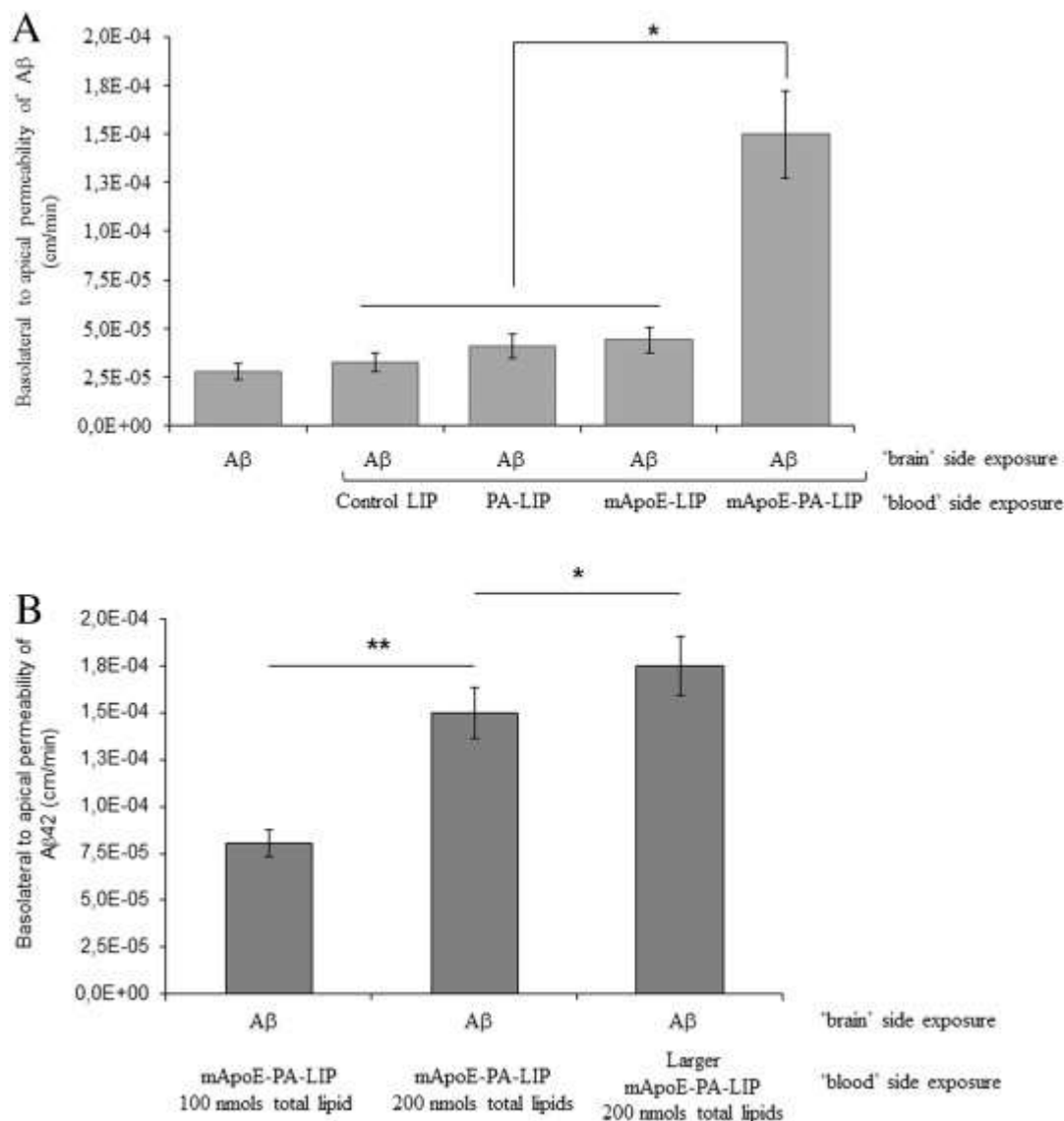

Figure 4. Aβ exchange across the BBB model in presence of liposomes.

hCMEC/D3 cells were cultured on transwell system and 500 nM of oligomers-enriched Aβ sample was added to the medium in the basolateral compartment and differently functionalized liposomes were added to PBS in the apical compartment. The amount of Aβ in the apical compartment of the

transwell system was measured by ELISA assay after different incubation times (0-240 min). **(A)** Endothelial permeability values of Aβ alone or in presence of 200 nmol (total lipids) of different liposomes. **(B)** Endothelial permeability values of Aβ in presence of different doses or size of mApoE-PA-LIP. * *P* < 0.05; ** *P* < 0.01.

Additional experiments were carried to evaluate the Aβo efflux from the brain side out when different doses of Aβo were added, at the beginning of the experiment, to mApoE-PA-LIP (200 nmol total lipids) present in the apical compartment. The results show that, under these conditions, the EP of the peptide was strongly reduced (Figure 5).
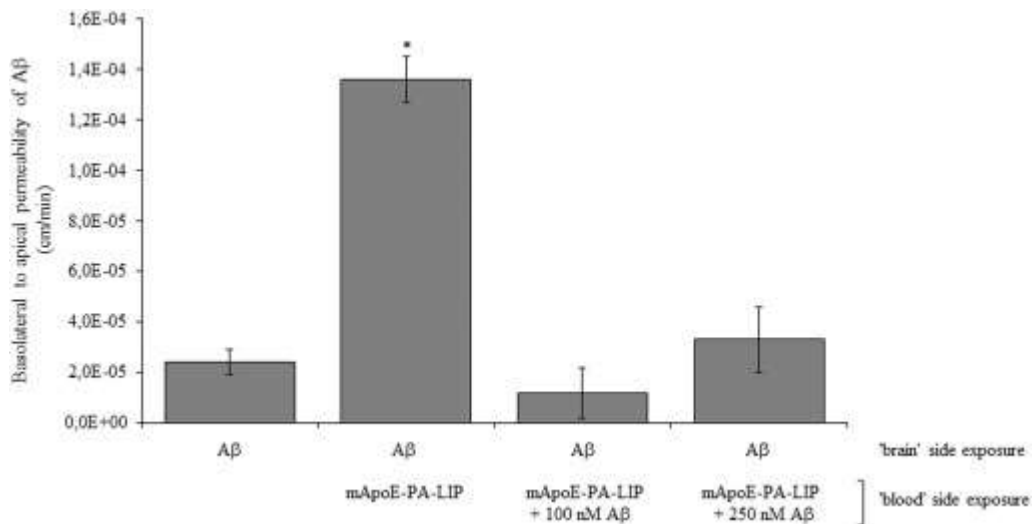

Figure 5. Aβ exchange across the BBB model in presence of liposomes/Aβo in the apical compartment.

hCMEC/D3 cells were cultured on transwell system and 500 nM of oligomers-enriched Aβ sample was added to the medium in the basolateral compartment and mApoE-PA-liposomes (200 nmol total lipids) were added to PBS in the apical compartment alone or together with different doses of Aβo. The amount of Aβ in the apical compartment of the transwell system was measured by ELISA assay after different incubation times (0-240 min) and the endothelial permeability values were calculated as described in the Methods section. * *P* < 0.05.

Since the ability of mApoE-PA-LIP to cross the BBB, even if low, has been shown both *in vitro* and *in vivo*,6, 22 we took into account the possibility that Aβo efflux is affected by the presence of mApoE-PA-LIP in the basolateral compartment. At first, we evaluated the effect of mApoE-PA-LIP of larger size (diameter = 426.2 ± 10 nm; PDI = 0.176; ζ-potential = − 16.33 ± 1.41 mV) with respect to the pores size (400 nm diameter) of the transwell filters on the exchange of Aβo across the BBB. Larger sized radiolabeled [³H]-sphingomyelin-mApoE-PA-LIP were added (200 nmol total lipids) to the apical compartment of the transwell and the radioactivity in the basolateral compartment was measured by liquid scintillation counting in order to verify their inability to reach the basolateral compartment. The results showed that after 2 h of incubation less than 0.1% of radioactivity present in the apical compartment was recovered in the basolateral one, confirming that these larger mApoE-PA-LIP were not able to cross the BBB. Successively, the efflux of Aβo from the basolateral compartment in the presence of these larger LIP in the apical compartment was measured by ELISA assay. The results showed that the Aβo EP was found to increase (+ 10%) in comparison with EP measured in the presence of smaller size mApoE-PA-LIP.

Secondly, we evaluated the effect of the presence of mApoE-PA-LIP in the basolateral compartment, together with Aβo, on peptide brain efflux. Radiolabeled [³H]-sphingomyelin-mApoE-PA-LIP were added (200 nmol total lipids) to the basolateral compartment of the transwell and the radioactivity in the apical compartment was measured by liquid scintillation counting. The results showed that only 0.043% of the total lipids was recovered in the apical compartment after 2 h of incubation (EP = $3.23 \pm 0.4 \times 10^{-6}$ cm/min), ruling out the possibility that LIP act as conveyor of Aβ.

Aβo clearance across the in vitro BBB model by mApoE-PA-LIP in physiological conditions

The effect of mApoE-PA-LIP on Aβo brain efflux was also evaluated under conditions simulating the protein concentration in blood[29] and CSF,[30] that are the presence of 4% BSA (w/v) in the apical compartment ('blood' side) and 0.015% BSA (w/v) in the basolateral one ('brain' side). The results (Figure 6) showed that also in these experimental conditions the basolateral-to-apical passage of Aβo was higher in the presence of mApoE-PA-LIP (EP = $4.30 \pm 0.3 \times 10^{-5}$ cm/min) in the apical compartment than in their absence (EP = $2.89 \pm 0.2 \times 10^{-5}$ cm/min).
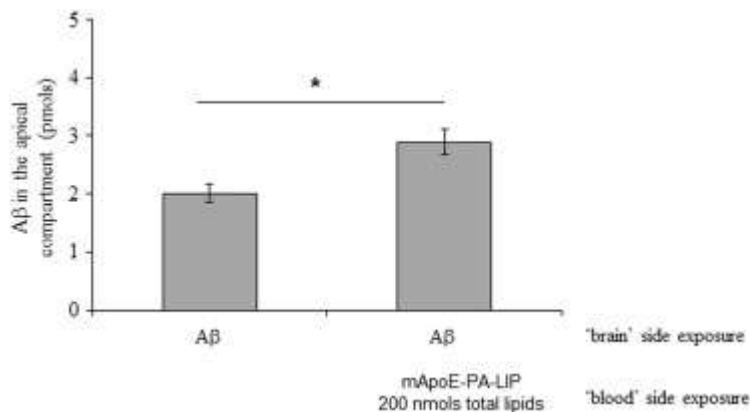

Figure 6. Aβ exchange across the BBB model in *in vivo*-like conditions.

hCMEC/D3 cells were seeded on a transwell system and 500 nM oligomers-enriched Aβ sample was added to a solution of PBS/0.015% BSA in the basolateral compartment and 200 nmol (total lipids) of mApoE-PA-LIP were added to PBS/4% BSA in the apical compartment. After 2 h of incubation the amount (pmol) of Aβ in the apical compartment of the transwell system was determined by ELISA assay. * $P < 0.05$.

Aβo clearance from the endothelial cells by mApoE-PA-LIP

We investigated the ability of mApoE-PA-LIP to draw out the peptide uptaken by hCMEC/D3 cells. For this purpose, the amount of Aβ uptaken by the cells from the basolateral compartment was measured by ELISA in the presence or absence of mApoE-PA-LIP (200 nmol total lipids) in the apical one. The results (Figure 7) showed that the amount of cell-associated Aβ decreased over time and, more importantly, the decrease was more pronounced in the presence of mApoE-PA-LIP in the apical compartment (Aβ clearance = 0.93 pmol/h) with respect to the peptide alone (Aβ clearance = 0.27 pmol/h). The results were also confirmed in *in vivo*-like conditions (data not shown) using PBS/BSA as described.
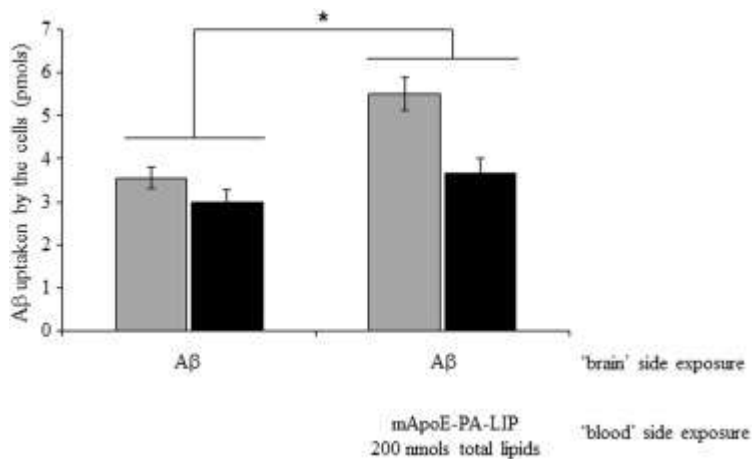
Figure 7. Aβ cellular uptake.

hCMEC/D3 cells were cultured on transwell system and 500 nM of oligomers-enriched Aβ sample was added to the medium in the basolateral compartment and 200 nmol (total lipids) of mApoE-PA-LIP was added to the PBS in the apical compartment. After 2 h (gray bars) or after 4 h (black bars) of incubation, cells were collected in a lysis buffer added with protease inhibitors and the Aβ content was measured by ELISA assay to determine the Aβ cellular uptake. * $P < 0.01$.

In vivo proof-of-concept

In order to assess the *in vivo* proof-of-principle, APP/PS1 mice (n = 7 mice/group; 16 months of age) were I.P. treated with 100 μl, 40 mM total lipids of mApoE-PA-LIP or with 100 μl of PBS (3 injections/week for 2 weeks). After treatment, mice were sacrificed and blood and brain were collected. Aβ levels in plasma and brain samples were quantified by ELISA assay. The results showed that plasma Aβ levels (Figure 8, A) of mApoE-PA-LIP treated mice (1.62 ± 0.66 pmol/ml) were significantly increased ($P < 0.05$, by Student's t test) as compared to PBS-treated mice (0.95 ± 0.48 pmol/ml). A decrease, not statistically significant, of brain Aβ levels (Figure 8, B) in mApoE-PA-LIP treated mice (3.53 ± 0.49 nmol/g brain) with respect to PBS-treated mice was detected (3.78 ± 0.54 nmol/g brain). The difference with respect to results previously reported[22] is probably due to the different treatment schedule used, as well as to the different age of animals.
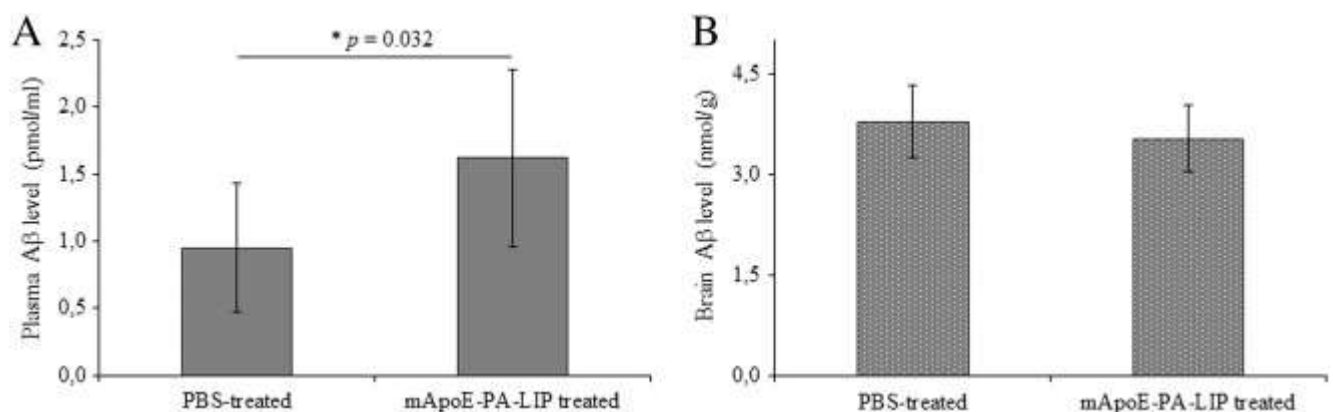

Figure 8. Aβ levels in APP/PS1 mice.

APP/PS1 (16 months of age) mice were intraperitoneally (I.P.) injected with mApoE-PA-LIP or with PBS as a control once every other day for two weeks (n = 7 mice/group). At the end of

treatment, animals were sacrificed and blood and brain were collected. Aβ levels were quantified by ELISA assays. **(A)** Plasma Aβ levels. **(B)** Brain Aβ levels. * $P < 0.05$.

Discussion

The clearance of Aβo, the most culprit Aβ assembly, from the brain and/or from cerebral blood vessels is considered a primary therapeutic target to counteract the onset/progression of AD.

As already suggested by Matzuoka et al.,[8] a possible strategy to reduce Aβo is to exploit the peripheral-sink effect using Aβ-binding agents. In the present investigation we evaluated the possibility to utilize nanoparticles6, 22 for this purpose, using an *in vitro* BBB transwell model and APP/PS1 as animal model of AD.

We chose mApoE-PA-LIP, that were previously showed to strongly reduce brain Aβ burden[22] when administered to AD mice. The further functionalization with mApoE increases the binding affinity of PA-LIP to Aβo, likely due to the existence of a synergic action of PA and mApoE as already suggested[6] and, even though it increases PA-LIP ability to reach the brain *in vivo*, this remains anyway low.6, 22 Therefore, it is possible that mApoE-PA-LIP efficacy *in vivo* is due their ability to withdraw Aβ peptide from the brain to the blood via sink effect.

First of all, we demonstrated the ability of mApoE-PA-LIP to bind either Aβo or Aβf and confirmed their already reported[6] ability to bind Aβ.

Sink effect *in vitro* investigations were performed on a BBB model made by hCMEC/D3 monolayer that separates two compartments: the basolateral one, mimicking the brain, and the apical one, mimicking the blood. Aβ samples were added to the basolateral compartment to resemble AD conditions. This model has been used herein not only as a prototype to study the effect of Aβ-binding particles on Aβ clearance from the brain across the BBB, as already proposed for Aβ-binding agents,[8] but also as a prototype to test the ability of Aβ-binding nanoparticles to remove Aβ from the endothelial cell monolayer. This makes the model useful as a tool to study potential therapeutic agents not only for AD, but also for CAA.

Initially, we verified the hypothesis by which Aβ is capable of moving across the BBB model establishing an equilibrium between 'brain' and 'blood' side.[31] Our results, showed that only small soluble Aβ assemblies, Aβo not Aβf, are spontaneously able to cross the endothelial monolayer. At the best of our knowledge, this is the first experimental demonstration of the assumption that large Aβ assemblies are not able to cross the endothelial cell layer used as a model of BBB.

In order to verify the hypothesized sink effect mechanism of action of mApoE-PA-LIP, Aβo efflux from the brain side was measured in presence of these liposomes in the apical compartment of the transwell system. The results here reported showed that in presence of mApoE-PA-LIP in the apical compartment, the permeability of Aβo from the basolateral compartment was strongly enhanced as compared to the presence of non- or mono-functionalized liposomes. The better performance of mApoE-PA-LIP with respect to other LIP is likely due to their higher binding affinity to Aβo.[6]

The effect of mApoE-PA-LIP on Aβo brain efflux was also evaluated under conditions simulating the different protein concentration in blood[29] and CSF.[30] Also under these conditions, an increase of Aβ EP was detected, even in a lesser extent, probably due to the formation of the so called 'protein corona', that might influence the mApoE-PA-LIP performance. This issue deserves further investigations.

Successively we took into account the possibility that Aβo efflux is affected by the presence of mApoE-PA-LIP in the basolateral compartment, since their ability to cross the BBB, even low, has been shown both *in vitro* and *in vivo*.[6, 22] Actually, some experiment carried out using liposomes of large size unable to cross the *in vitro* BBB model showed an increased Aβo efflux. This suggests that the passage of mApoE-PA-LIP to the basolateral compartment could limit Aβ efflux, likely due to their binding avidity for Aβ. Further experiments, showing that mApoE-PA-LIP passed to the basolateral compartment were not able to turn back to the apical one, ruled out also the possibility that they may act as Aβo carrier from the brain to the blood.

In order to better clarify the mechanism underlying the increased Aβ efflux exerted by mApoE-PA-LIP in the apical compartment, experiments were carried out in the presence of peptide together with LIP. A strong reduction of peptide efflux from the brain side under these conditions was observed, likely due to the decreased ability of mApoE-PA-LIP, already engaged with Aβo added to the blood side, to withdraw new peptide arriving from the brain side.

All these results support the hypothesis that oligomers spontaneously cross the barrier model and, secondarily, they are caught by the LIP in the apical compartment. Then, the removal of Aβo by LIP is likely shifting the equilibrium of the peptide across the barrier toward the apical compartment, eliciting its efflux.

Taken together, these results suggest that mApoE-PA-LIP could exert the sink effect also *in vivo*. As a proof of this possibility, *in vivo* investigation showed that peripheral administration of mApoE-PA-LIP to transgenic APP/PS1 mice significantly increased plasma Aβ levels.

Finally, we took into account another aspect implicated in the development and/or progression of AD[32]: the deposition and aggregation of Aβ within the walls of the cerebral vasculature. Thus, we investigated the ability of mApoE-PA-LIP to draw out the peptide uptaken by hCMEC/D3 cells. The results showed that in the presence of mApoE-PA-LIP the amount of cell-associated Aβ decreased. This might open a parallel scenario concerning the possible use of mApoE-PA-LIP, not only for AD, but also as a potential treatment for CAA.

In conclusion, in the future outlook to design AD therapies, these results highlight the importance of designing multifunctional nanoparticles able to sequester peripheral blood Aβ (without the need to cross the BBB), taking into account the necessity to disaggregate brain Aβ aggregates to make Aβo available. It is also important to point out that also the alterations reported at charge of the BBB in AD,[33] should be considered.

Figure S1. Characterization of Aβ samples.

(A) Aβ$_{1-42}$ samples were analyzed by SDS-PAGE electrophoresis gel and immunoblotted with anti-Aβ$_{1-42}$ 6E10, followed by ECL detection. Aβ preparation enriched in monomers (lane a) or in small aggregates (lane b) are shown. Molecular weights are indicated. **(B)** AFM analysis of Aβ oligomers-enriched preparation. **(C)** AFM analysis of Aβ fibrils-enriched preparation. Representative 4 × 4 μm AFM images of Aβ are shown. Bars: 500 nm.

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