Research paper

Anti-Aβ-MAb and dually decorated nanoliposomes: Effect of Aβ1-42 peptides on interaction with hCMEC/D3 cells

Eleni Markoutsa a, Konstantina Papadia a, Carla Clemente b, Orfeu Flores b, Sophia G. Antimisiaris a,c,*

a Laboratory of Pharmaceutical Technology, Department of Pharmacy, University of Patras, Rio, Greece
b STAB VIDA, Investigação e Serviços em Ciências Biológicas Ltda, Caparica, Portugal
*c Institute of Chemical Engineering and High Temperatures, FORTH/ICE-HT, Rio, Greece

ARTICLE INFO

Article history:
Received 28 November 2011
Accepted in revised form 10 February 2012
Available online xxxx

Keywords:
Targeting
Amyloid
Abeta peptides
RAGE
Blood–brain barrier
Transcytosis

ABSTRACT

Anti-Aβ-MAb (Aβ-MAb)-decorated immunoliposomes (LIP) and dually decorated ones (dd-LIP) with OX-26 and Aβ-MAb were constructed. In both cases, the biotin-streptavidin ligation method was applied. All LIP types were characterized for size distribution, zeta potential, and integrity during incubation with serum proteins. Uptake and transcytosis of both LIP types and control vesicles by human brain endothelial hCMEC/D3 cells were measured. All LIP types had mean diameters below 150–200 nm and low polydispersity. Aβ-MAb-LIP uptake was higher than control PEGylated liposomes, while uptake of dd-LIP was similar to that of OX-26-LIP. Aβ-MAb-LIP and dd-LIP uptake increased significantly when cells were pre-incubated with Aβ1-42 peptides; OX-26-LIP uptake was not modulated. Transcytosis of Aβ-MAb-LIP through monolayers was 2.5 times higher when monolayers were pre-incubated with Aβ1-42. Transport of both probes, FITC-dextran and rhodamine-lipid, was equivalent, indicating that Aβ-MAb-LIP are transferred intact through the BBB model. The Aβ peptide-induced increase in binding (and transport) is regulated by the membrane receptors for Aβ1-42 peptides (RAGE), as proven after blocking RAGE by a specific MAb. Aβ1-42 peptides did not modulate the barrier tightness and integrity, as determined by transendothelial resistance and Lucifer Yellow permeability. Additionally, hCMEC/D3 cell viability was not affected by Aβ peptides or by Aβ-MAb-LIP.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Over the last decade, nanoparticle-mediated drug delivery represents a promising strategy to increase the CNS penetration of several therapeutic moieties [1–3]. Indeed, a number of different nanocarrier types (as liposomes, nanoparticles, nanocapsules, etc.) are being investigated for diagnosis and/or treatment of brain-located pathologies as various types of cancer and neurological disorders, as Alzheimer’s disease (AD) [4,5]. Several of the most recent approaches implicate multifunctional nanoparticles in order to achieve increased targeting potential [6,7].

AD is the most common form of dementia in the elderly, with no therapy or definite diagnosis, at the time. In the last years, many efforts toward AD therapy have focused on the “amyloid hypothesis” that aims at preventing Aβ formation, blocking its aggregation into plaques, or lowering the levels of soluble Aβ in the brain and perhaps also disassembling existing aggregates [8–10]. The recently initiated nanotechnological approaches for therapy and/or diagnosis of AD have considered a number of ligands to target nanoparticles to the BBB receptors (as endogenous and foreign proteins, antibodies, and peptides derived from protein binding domains) [1,5,6]. Furthermore, additional ligands have been proposed as potential moieties to target amyloid species (as different types of lipids [11], curcumin or curcumin derivatives [10,12,13], Aβ peptide specific MAb’s [14,15], etc.). Within this frame, we have recently designed nanoliposomes (NL) decorated with an anti-Aβ1-42 monoclonal antibody (Aβ-MAb) that demonstrated very high affinity for Aβ1-42 peptides, in vitro [14]. In the present investigation, we explore the possibility to enhance the interaction of such immunoliposomes (LIP) with brain capillary endothelial cells, as a methodology to enhance their passage across the BBB. We describe the preparation and physical characterization of dually decorated immunoliposomes (dd-LIP) with Aβ-MAb together with a monoclonal antibody against the transferrin receptor (TIR), OX-26, recently demonstrated to increase lysosome interaction (compared to control liposomes), with human immortalized brain capillary cells (hCMEC/D3) cultured in vitro [16]. The interaction of the constructed liposomes with the hCMEC/D3 cellular model of BBB was investigated.

It is known from the relevant literature that a specific receptor, the receptor for advanced glycation end products (RAGE), is involved in the transcytosis of Aβ peptides across the BBB (from
the blood to the brain) [17,18], and the increased expression of RAGE in Alzheimer’s disease brain has been related to the pathogenesis of neuronal dysfunction [17]. In order to investigate the potential role of the RAGE receptor on the transport of Aj1-MAB-liposomes across a monolayer of hCMEC/D3 cells, the interaction was studied in absence and presence of Aj1-42 peptides and/or an anti-RAGE MAb. In all experiments, control liposomes, decorated with nonspecific antibody, or not (plain PEGylated liposomes), were also studied under identical conditions.

2. Materials and methods

1,2-Distearoyl-sn-glycerol-3-phosphatidylcholine (DSPC), 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene)glycol]-2000 (PEG-lipid), 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine-N-[biotinyl(polyethylene)glycol]-2000 (Biotin-lipid), and lissamine rhodamine B phosphatidylethanolamine (RHO-lipid) were purchased from Avanti Polar Lipids. Fluorescein-isothiocyanate-dextran-4000 (FITC-dextran), streptavidin from Streptomyces avidinii, lucifer yellow-CH dilithium salt (LY), IgG from murine serum (mouse IgG), Sephadex G-50, and Sepharose CL-4B were from Sigma–Aldrich. Anti-transferrin receptor (TIR) monoclonal antibody was obtained by Serotec. An anti-Aj1-42 monoclonal antibody (Aj1-42-MAb) was produced by Stab Vida, as previously described [14]. A monoclonal Anti-human RAGE antibody (RAGE MAb) was purchased from R&D Systems (clone 176902). When needed, antibodies were biotinylated using the EZ-link Biotinylation kit (Pierce). In brief, 1 mg of antibody was allowed to react with the biotinylation reagent (Sulfo-NHS-LC-Biotin) at a molar concentration ratio (reagent/antibody) 20:1 in 1 ml reaction volume for 2 h at 4°C. Biotin-antibody was purified from non-reacted reagent with Zeba desalt spin columns (Pierce).

Amicon-Ultra 15 ultrafiltration tubes (Millipore) were used for sample concentration, after elution from gel-filtration columns. Protein concentrations were measured, when needed, by the Bradford microassay (Biorad), using bovine serum albumin as standard. Aj1-42 and Aj1-40 peptides were purchased from Mario Negri Institute, IT. All other chemicals were of analytical grade and were obtained from either Sigma–Aldrich or Merck. Materials used for cell culture studies are mentioned below.

Fluorescence intensity (FI) of samples was measured with a Shimatzu RF-1501 spectrophuorometer at 37 ± 0.1°C. The following conditions were used for measurement of each specific dye: (i) For Rho-lipid, EX-540 nm/EM-590 nm; (ii) For FITC, EX-490 nm/EM-525 nm. In all cases, 5 nm slits were used.

A bath sonicator (Branson) and a probe sonicator equipped with a microtip (Vibra-cell, Sonics and Materials Inc.) were used for liposome preparation.

2.1. Immuno-liposome preparation

Liposomes consisting of PC or DSPC/Chol/DSPE-PEG2000/DSPE-PEG2000-Biotin at 20:10:0.80:0.015–0.0065 (mole ratios) with surface biotin anchors were prepared by the thin film hydration method, as previously described [14,16]. The Biotin-lipid amount added in the lipid mixture was modulated, in order to have lower or higher density of MAb on the vesicle surface. In some cases, plain PEGylated liposomes (with identical lipid compositions but no Biotin-lipid) were prepared as control vesicles. 36 mM FITC-dextran was entrapped in vesicles (osmolarity always adjusted to 300 mOsM with NaCl (Roebbling osmometer)). In some cases, vesicles were additionally labeled with RHO-lipid. After initial formation, liposome dispersions were homogenized by probe sonication until they became clear and centrifuged (12,000 rpm, 10 min) in order to precipitate and discard large liposomal aggregates and/or titanium particles that leaked from the probe. Finally, liposome dispersions were incubated for 1 h at 55°C for DSPC-containing liposomes, in order to anneal structural defects. Free FITC-dextran or HPTS was separated from liposomes by size exclusion chromatography (Sepharose 4B-CL (1 x 35 cm) column).

For conjugation of antibodies to liposomes, the biotinylated liposomes were initially incubated in the presence of 10-fold molar excess STREP (compared to surface exposed biotin) for 1 h at 25°C and then overnight at 4°C. Free STREP was removed by a Sepharose 4B-CL column. IgG or Ox-26 decoration of biotinylated-STREP-labeled liposomes was performed by incubating liposomes (at a lipid concentration of 0.5 mg/ml) with an excess of the appropriate (in each case) biotin-antibody, at 25°C for 1–2 h and then overnight at 4°C. Non-attached antibody was removed by gel filtration (Sephadex 4B-CL column) and collected for measurement of antibody attachment yield. For this free biotinylated MAB’s in collected fractions were quantified by an ELISA technique; in brief, polystyrene 96-well plates were coated with STREP (2 μg) for 16 h at 4°C. After washing with PBS and blocking with 2% bovine serum albumin (BSA) (1 h, 37°C), samples or known concentrations of biotinylated MABs (for quantification) were applied (1 h at 37°C) and well plates were washed with PBS. Rabbit anti-mouse IgG labeled with peroxidase (Sigma) was added as second antibody (at 1:2000 dilution in 1% BSA) for 1 h at 37°C, and wells were washed with PBS (×3) and with 0.1 M citrate buffer (×1) and incubated at RT with 100 μl peroxidase substrate solution (0.06% H2O2 and 18.5 mM o-phenylenediamine) until color development (10 min). Reaction was stopped with sulfuric acid, and OD at 492 nm was measured. Appropriate calibration curves were constructed using known concentrations of MABs.

2.2. Lipid physicochemical characterization

2.2.1. Vesicle entrapment efficiency

The dye/lipid (mol/mol) ratio was calculated in all liposome types in order to be able to calculate the amount of lipid from the corresponding dye concentration. For this, the FI of the samples was measured at the specific EM and EX wavelength maxima for each dye (see above). The lipid concentration of the liposome dispersions was measured by the Stewart colorimetric assay [19].

2.2.2. Size distribution and zeta potential measurements

Particle size of diluted (with PBS pH 7.40) vesicle dispersions (0.4 mg/ml) was measured by dynamic light scattering (DLS) technique (Malvern Nano-Z, Malvern Instrument, UK) at 25°C at a 173° angle. In some cases, the size distribution was measured during extended time periods of incubation for estimation of LIP colloidal stability. Zeta Potential was measured for the same samples (dispersed in 10 mM PBS, pH 7.40) at 25°C, by the same instrument (utilizing the Doppler electrophoresis technique).

2.2.3. Liposome integrity

The integrity of liposomes was studied by measuring the retention of liposome-entrapped fluorescent dye (calcine [100 mM]) during incubation in buffer, or PBS, at a final lipid concentration of 0.4 mg/ml. For this, 1 volume of liposome dispersion was mixed with 5 volumes of media (buffer or PBS) and incubated at 37°C for 48 h. Calcein latency was measured at 0, 2, 4, 6, 24, and 48 h, as reported before [20].

2.3. Cell culture

Immortalized human brain capillary endothelial cells (hCMEC/D3) between passage 25 and 35 were used in all studies. The hCMEC/D3 cell line was obtained under license from Institute national de la Sante et de la Recherche Medicale (INSERM, Paris, Conclusion and Discussion

The study demonstrated that liposomes decorated with RAGE antibodies could be used to target specific brain endothelial cells, specifically hCMEC/D3 cells. The RAGE receptor on the surface of these cells facilitates the uptake of liposomes, allowing for targeted delivery of therapeutic agents to the brain. The use of biotinylated liposomes and RHO-lipid for conjugation and labeling, respectively, provided a means to optimize the efficiency of liposome attachment and to study the specific interactions between the liposomes and the RAGE receptor.

The co-incubation of liposomes with non-specific antibodies was utilized to assess the specificity of the RAGE-mediated uptake, highlighting the importance of RAGE in the process. The results showed a significant increase in liposome accumulation in the presence of RAGE-specific antibodies, indicating the potential for targeted drug delivery to the brain.

Furthermore, the biotin-avidin system was employed to tether liposomes to the surface of hCMEC/D3 cells, demonstrating the feasibility of tethering liposomal drug delivery systems to brain endothelial cells. This approach could potentially be used to deliver therapeutic agents to specific regions of the brain, with potential applications in the treatment of neurodegenerative diseases such as Alzheimer’s.

Despite the promising results, further studies are needed to optimize liposome formulation and to evaluate the therapeutic potential of RAGE-targeted liposomes in animal models of neurological conditions. Ongoing research is focused on improving the stability and controlled release of liposomes, as well as investigating the long-term effects of RAGE-targeted liposome delivery on brain integrity and function.
France). Cells were seeded in a concentration of 27,000 cells/cm² and grown in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 10 mM HEPES, 1 ng/ml basic FGF (bFGF), 1.4 μM hydrocortisone, 5 μg/ml ascorbic acid, penicillin–streptomycin, chemically defined lipid concentrate, and 5% (ultra-low IgG) FBS. The cells were cultured at 37 °C, 5% CO₂/saturated humidity. All culture wares were coated with 0.1 mg/ml rat tail collagen type I. Liposomes and grown in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 10 mM HEPES, 1 ng/ml basic FGF (bFGF), 1.4 μM hydrocortisone, 5 μg/ml ascorbic acid, penicillin–streptomycin, chemically defined lipid concentrate, and 5% (ultra-low IgG) FBS. The cells were cultured at 37 °C, 5% CO₂/saturated humidity. All culture wares were coated with 0.1 mg/ml rat tail collagen type I.

2.4. LIP uptake by cells

For the study of LIP and dd-LIP uptake by cells, vesicles (immunoliposomes or control liposomes) that were labeled with FITC-dextran in their aqueous compartments were incubated with confluent monolayers of hCMEC/D3 cells (100–600 nmoles liposomal lipid/10⁶ cells) in cell culture medium at 37 °C, for different time periods (15 or 60 min). In concentration–response experiments, different amounts of liposomes were incubated with cells for 1 h. After incubation, the cells were washed in ice-cold PBS (pH 7.4), detached from plates by scraping, re-suspended in 1 ml of PBS and assayed by FI measurements (after cell lysis in 2% Triton X-100). The auto fluorescence of cells was always subtracted.

In some cases, LIP–cell interactions were evaluated in presence of Aβ1-42 or Aβ1-40 peptides, which were disaggregated immediately before each study, by an age reversal protocol described previously [21]. Aβ1-42 peptides were used at 100 nM concentration, molar excess of the Aβ1-42-MAb on the LIP surface (the amounts used ranged between 0.7 and 3.3 μg per well). Three different protocols were tested: [P-1] Aβ-peptides and liposomes were incubated with the cells concurrently (in cell culture medium or HBSS); [P-2] Cells were pre-incubated with Aβ1-42 peptides for 10 min, cell medium was replaced, and liposomes were added; and [P-3] Liposomes were pre-incubated with Aβ1-42 peptides for 10 min, and the mixture was incubated with the cells.

In separate sets of experiments, the uptake of LIPs by cells was evaluated after pre-incubating the cells with RAGE MAb (5 μg/ml) for 15 min.

2.5. Cell monolayer permeation studies

The transport of Aβ1-MAB-LIP through hCMEC/D3 cell monolayers was studied in absence and presence of Aβ1-42 peptides. For this, cells were seeded on type I collagen pre-coated transwell filters (polycarbonate 6 well, pore size 0.4 μm; Millipore), as previously described [16,22], and 24 h before each transport experiment, the medium was replaced with fresh containing 1 nM sinnvastatin, for the formation of tight junctions [21,22]. The cell monolayer was periodically inspected under a microscope, and the transendothelial electrical resistance (TEER) was monitored during the experiment. The quality of the monolayers was also tested by measuring the permeability of Lucifer yellow (LY), a highly hydrophilic low-molecular-weight test compound (MW 457.25), as described before [23,24]. Transport experiments were conducted in cell culture medium. The transport was estimated by placing Aβ1-MAB-LIP with FITC-dextran encapsulated and RHO-Lipid incorporated in the vesicles on the upper side of the monolayers (200 nmol of lipid per well) and measuring FI of both FITC and RHO, in the well media at various time periods (20, 40, and 60 min). The RHO-Lipid was extracted from samples (0.5 ml HBSS) from the lower compartments of the transwell system with 2 ml of CHCl₃/MeOH (2:1) and applying vortex for 2 min. Samples were then centrifuged (3 min at 3000 rpm), aqueous phase was removed, and FI of the organic phase was measured.

In separate sets of experiments, the transport of Aβ-MAB-LIP through hCMEC/D3 cell monolayers was evaluated after pre-incubating the monolayers with RAGE MAb (20 μg/ml) for 15 min. In order to investigate whether the presence of Aβ-peptides disrupts or modifies the monolayer barrier (due to toxicity), lucifer yellow (LY) was used as a control for paracellular transport of the monolayers. For this, LY permeability was calculated on hCMEC/D3 monolayers that were incubated with Aβ1-42 peptides, at the peptide/ cell loads, which were used in the transport studies and others that were not (control). Additionally, the TEER of both sets of monolayers was monitored during the experiment.

2.6. Cytotoxicity assay

Cytotoxicity assays were performed in order to evaluate whether Aβ-MAB-LIP, Aβ1-42 peptides, or their mixtures exacerbated toxicity to the cells, at the conditions used in the cell culture studies. Cells were grown on 24-well plates until confluent. Medium was replaced and LIP, or Aβ1-42 peptides, or mixtures were incubated with cells for 24 h at 37 °C, 5% CO₂/saturated humidity. After incubation, the medium was removed and the cells were washed with PBS. Fresh medium containing 0.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. Cells were incubated for 2.5 h, the medium was removed, and DMSO was added (at 37 °C for 30 min) to dissolve the formazan crystals that formed. Alive cells (%) were calculated by the formula

\[
\frac{A570_{	ext{control}} - A570_{	ext{background}}}{A570_{	ext{sample}} - A570_{	ext{background}}} \times 100
\]

where A570_control is the OD-570 nm of untreated cells and A570_background the OD-570 nm of MTT without cells. Triton X-100 surfactant was used as a positive control of cytotoxicity.

2.7. Statistical analysis

Each experiment included at least triplicate wells for each condition tested. All results are expressed as mean ± SD from at least three independent experiments. The significance of variability between results from various groups was determined by one-way analysis of variance.

3. Results

3.1. LIP physicochemical characteristics

The theoretical structure of the dd-LIPs constructed is shown in Fig. 1. All types of liposomes had mean diameters below 200 nm (Table 1) and very low polydispersity indices (always <0.200), indicating a narrow size distribution. In comparison with plain pegylated liposomes (PEG–liposomes), the immunoliposomes were always slightly larger, as anticipated due to the surface immobi-
lized antibody (or antibodies). In fact, dd-LIP had the highest mean diameter, compared to all other LIP types studied.

The MAb attachment yield on LIPs was always higher than 85% (compared to the anticipated theoretical value), as calculated by Elisa technique. As seen in Fig. 2, all the types of liposomes investigated are stable and retain a high fraction of their content (>95% of initially encapsulated calcine) during 48 h of incubation in buffer. When incubated in presence of serum proteins, both types of immunoliposomes (dd-LIP [in Fig. 2A] and Aβ-MAb-LIP [in Fig. 2B]) were seen to gradually lose some of the encapsulated dye; however, the latency was higher than 60% (of initial) after 24 h of incubation in presence of serum proteins, for all liposomal formulations evaluated. The dd-LIP were seen to be more stable (calcine latency was higher than 85% (of initial) after 24 h incubation (Fig. 2A)), compared to the Aβ-MAb-LIP (Fig. 2B), demonstrating the in vivo applicability of the dual targeted formulations, as also verified before [16] for ox-26 LIPs.

FITC-dextran-encapsulating liposomes (as anticipated since FITC-Dextran is a larger molecule compared to calcine and is expected to be retained at higher amounts in the vesicles) were also demonstrated to be very stable under identical conditions with those used in the cell culture studies (results not shown). Thereby, it is proven that during incubation of the current liposome types with cells, encapsulated dyes will not leak out from the vesicles, and thus, dye uptake will be equivalent to vesicle uptake.

### 3.2. Immunoliposome–cell interactions

#### 3.2.1. Uptake of Aβ-MAb-LIP and dd-LIP by cells

The uptake of Aβ-MAb-LIP by hCMEC/D3 cells is significantly higher compared to control vesicles, however lower than that of ox-26 LIPs (Fig. 3). However, it should be emphasized that the density of MAb on the surface of LIPs was about three times lower in the case of the Aβ-MAB (0.015 mol% of total lipid) compared to that of ox-26 (0.05 mol%). The uptake of ox-26 LIPs measured herein was similar to that measured recently [16] under identical incubation conditions. Interestingly, when the two antibodies are attached on the surface of the same vesicle, the uptake of the dd-LIP by hCMEC/D3 cells is similar to that measured with the ox-26 LIPs.

Although the mechanism by which the Aβ-MAB-LIPs are taken up by hCMEC/D3 cells is not known, the amount of LIPs taken up by the cells is the same when 200, 300, or 400 nmol of LIPs is incubated with 10⁶ cells, indicating that the mechanism for the uptake of Aβ-MAB-LIPs is saturated at the lipid dose of 200 nmol lipid/10⁶ cells (Fig. 4B). Additionally, the interaction is rapid, since the same uptake was measured after 15 and 60 min of incubation (Fig. 4A), and already at 15 min, a considerable amount of liposomal lipid is taken up by the cells.

#### 3.2.2. Effect of Aβ1–42 peptides on interaction with hCMEC/D3 cells

When performing the uptake study in presence of Aβ1–42 peptides (0.7 μg), the uptake of Aβ-MAB-LIPs and dd-LIPs was substantially increased (Fig. 5). Indeed the uptake was increased by 59% and 24% for Aβ-MAB-LIPs and dd-LIPs, respectively. Oppositely, the uptake of all the control liposome types tested was not at all affected by Aβ-peptides. For ox-26 LIP, on the other hand, the presence of Aβ1-42 peptides resulted in a slight (nonsignificant) decrease in uptake. Although the reduction was not statistically significant, it may be postulated that the presence of the peptides blocks the ox-26-decorated immunoliposomes from approaching the Tf receptors on the cell membranes.

Aβ1-42 peptides were also demonstrated to increase the interaction of Aβ-MAB-LIP with cells (results not shown) when different lipid doses of the LIPs were incubated with the cells (300 or 400 nmol of lipid/10⁶ cells) or when lower incubation periods were used (15 min). Similar results were obtained when Aβ1-40 peptides were used instead of Aβ1-42 (not shown). Additionally, when the protocol of incubation was modified by pre-incubating the peptides with the cells for 10 min, adding fresh medium and then adding the LIPs, the uptake of Aβ-MAB-LIP was increased by 90%. However, when the LIPs were pre-incubated for 10 min with the peptides, before the mixture was incubated with the cells, the effect of the peptides was abolished and uptake of LIPs by the cells was not modified by the peptides. Perhaps this last result indicates

---

**Table 1**

<table>
<thead>
<tr>
<th>Liposome type</th>
<th>Size (nm)</th>
<th>PI</th>
<th>Z-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>128.6 ± 2.1</td>
<td>0.195</td>
<td>−2.33 ± 0.20</td>
</tr>
<tr>
<td>Anti-Aβ</td>
<td>147.6 ± 8.7</td>
<td>0.170</td>
<td>−2.70 ± 0.99</td>
</tr>
<tr>
<td>Ox-26</td>
<td>153.3 ± 9.1</td>
<td>0.141</td>
<td>−3.6 ± 1.9</td>
</tr>
<tr>
<td>Anti-Aβ/Ox-26</td>
<td>165.8 ± 1.7</td>
<td>0.176</td>
<td>−4.0 ± 1.6</td>
</tr>
</tbody>
</table>

---

**Fig. 2.** Latency of calcine (%) in the various types of immunoliposomes (dd-LIP [A] and Aβ-MAB-LIP [B]) and their corresponding controls (decorated with the same amounts of IgG) during 48 h incubation in presence of buffer or FCS (80% v/v), at 37 °C. Final lipid concentration was always 0.4 mg/ml. Each value is the mean of 4 different samples, and bars represent standard deviations of each mean value. The symbol keys are included in the graph inserts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 3.** Uptake of ox-26 LIP; Aβ-MAB-LIP and dd-LIP (with ox-26 + Aβ-Mab), and corresponding control liposomes (200 nmol of lipid in all cases) by 10⁶ hCMEC/D3 cells at 37 °C after 60 min incubations. Aβ-MAB-LIP (and IgG low) contained 0.015 mol% BioIn lipid (of total lipid) on their surface, while ox-26 LIP (and IgG high) 0.05 mol% (equivalent to MAb amount). Uptake is expressed as nanomoles of lipid associated with cells after incubation with liposomes. Each value is the mean of at least 3 independent experiments, and the SDs of means are presented as bars ("p < 0.05; **"p < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that the peptides bind to the Aβ-MAB-LIP surface before they can reach possible receptors on the cell membranes and thus do not induce increased liposome uptake.

Interestingly, when the interaction between Aβ-MAB-LIPs and hCMEC/D3 cells is studied in presence of HBSS instead of cell culture medium (which includes 10% v/v FCS), the uptake of the LIPs is decreased to approximately half (0.1819 ± 0.0092 nmole lipid, compared to 0.46 ± 0.12) approaching the uptake values measured when medium was used in the donor phase (1.4–1.68 × 10⁻³ cm/min, in very good agreement with the previously reported value (1.33 × 10⁻³ [24]) and with the values measured when medium was used in the donor phase (1.4–1.68 × 10⁻³ cm/min), which are slightly higher due to a difference in tight junction formation, as demonstrated also by others [23,24]. From the results (presented in Fig. 6), it is clear that when Aβ1–42 peptides are pre-incubated with the cell monolayer, the transport of the Aβ-MAB-LIP is substantially increased by between 1.5 and 3.35 times. The differences between transport in absence and in presence of Aβ1–42 peptides are always statistically significant and take place very fast even after only 20 min. Interestingly, at longer time periods, and especially after 60 min, the effect of the peptides on

to take place. Perhaps this is linked with the fact that abeta peptides are normally bound to albumin and/or specific lipoproteins in human plasma under physiological conditions and transported as complexes [25].

3.3. Aβ-MAB-LIP transport studies

In order to investigate whether the Aβ1–42 peptide-induced increase in the interaction between hCMEC/D3 cells and Aβ-MAB-LIPs will also have an effect on the transport of the liposomes across a monolayer of cells – which is a good model for nanoparticle transport across the BBB, as previously seen [16,26] – immuno-liposome transport experiments were performed using transwell cultured cell monolayers. Cell culture medium was used in the donor phase of the filter system and HBSS in the receiving compartment, in order to have similar conditions with those of the interaction studies in which peptides were shown to increase Aβ-MAB-LIP/cell interaction. All other conditions were identical with those used previously. The TEER was 60.0 ± 8.9 Ω cm⁻², similar to the value measured previously (63.9 ± 5.5 Ω cm⁻², [16]). The permeability of LY was measured and found to be 1.47 × 10⁻³ ± 0.00012 cm/min, in very good agreement with the previously reported value (1.33 × 10⁻³ [24]) and with the values measured when medium was used in the donor phase (1.4–1.68 × 10⁻³ cm/min), which are slightly higher due to a difference in tight junction formation, as demonstrated also by others [23,24]. From the results (presented in Fig. 6), it is clear that when Aβ1–42 peptides are pre-incubated with the cell monolayer, the transport of the Aβ-MAB-LIP is substantially increased by between 1.5 and 3.35 times. The differences between transport in absence and in presence of Aβ1–42 peptides are always statistically significant and take place very fast even after only 20 min. Interestingly, at longer time periods, and especially after 60 min, the effect of the peptides on
Aβ-Mab-LIP transport is lower (uptake increases by 1.5–1.8 times) compared to that at 20 min (uptake increases by 2.8–3.4 times), indicating that the transport mechanism which is influenced by Aβ peptides is of high velocity.

As seen (Fig. 6), the amounts of lipid transported are similar when they are calculated based on the transport of the hydrophilic label (FITC-dextran) or that of the lipophilic label (RHO-lipid), and FITC/RHO ratios are always close to 1 (Table 2), indicating that most likely intact vesicles are transported across the monolayer.

In order to rule out the possibility that perhaps the increased translocation of LIPs after pre-incubation of the monolayer with Aβ peptides is due to a disturbance of the barrier by the peptides, the monolayer TEER and also the permeability of LY were measured also in the presence of peptides (using identical conditions as those applying in the transport studies). As seen in Fig. 7, there is no significant difference in the LY permeability values measured in both cases (Fig. 7A). Also, the TEER generated by the monolayer is not affected by the peptides and remains steady during the duration of the transport experiment (Fig. 7B).

### 3.4. Inhibition of Aβ-Mab-LIP uptake and transport by RAGE Mab

In order to investigate whether the receptor primarily responsible for the transport of Aβ across the BBB (receptor for advanced glycation end products, RAGE) [17,18] is implicated in the increased uptake of Aβ-Mab-LIP (compared to IgG-immunoliposomes) and the effect of Aβ peptides on the uptake/transport of these liposomes by the hCMEC/D3 cells, we conducted uptake and transport experiments in which we pre-incubated the cells with RAGE MAb. As seen in Fig. 8, both the uptake (A) and the transport (B) of Aβ-Mab-LIP are decreased when the cells are pre-incubated with the RAGE specific MAb. In fact (Fig. 8A), Aβ-Mab-LIP uptake was demonstrated to increase significantly in presence of two times molar excess and (even more) 10 times molar excess (compared to the Aβ-Mab on the LIP surface) Aβ peptides, and in both of these cases, the uptake was reduced by approximately 15% when the cells were pre-incubated with 5 μg/ml or RAGE MAb. Nevertheless, inhibitions were not statistically significant (p > 0.05), most possibly due to the low MAb concentration used [27]. When Aβ-Mab-LIP transport was conducted after pre-incubating the cell monolayers with 20 μg/ml RAGE MAb (Fig. 8B), the LIP transport was substantially reduced, in absence (significant reduction for the 60 min time point) and also in presence of Aβ peptides (significant reduction for both time points used). In fact, the transport value measured in presence of Aβ peptides plus RAGE MAb (presented as colored and patterned bars in the graph) was equal to the corresponding control value (no peptides and no inhibitor) at the 20 min time point, but significantly lower than the control at 60 min, which correlates well with the fact that at the latter time point, the LIP uptake is significantly reduced by RAGE MAb (also in absence of peptides). The results presented are based on measurements of FITC-dextran FI; however, the values calculated by measuring RHO-lipid FI (not shown) were similar. Therefore, it is clearly shown that RAGE is directly implicated in the Aβ peptide-induced increased transport of the Aβ-Mab-LIP, as well as in the increased interaction of these LIPs with hCMEC/D3 cells (compared to control immunoliposomes) in absence of any peptides.

### 3.5. Cell toxicity studies

Control liposomes and immunoliposomes were tested for their cytotoxicity toward hCMEC/D3 cells, after 24 h of incubation with the cells, by the MTT assay. Incubations were performed in absence

---

**Table 2**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Incubation period (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Aβ1-42</td>
<td>0.77</td>
</tr>
<tr>
<td>No peptides</td>
<td>0.91</td>
</tr>
</tbody>
</table>

---

and in presence of Aβ1-42 peptides, at the same peptide–cell ratios applying in the liposome–cell interaction studies. Results (Fig. 9) prove that Aβ-MAb-LIPs as well as corresponding control liposomes are nontoxic for the cells, in absence and also in presence of Aβ1-42 peptides (at the same concentrations as those used in the uptake studies). Additionally, Aβ1-42 peptides did not cause any reduction in cell proliferation, when incubated with the cells at concentrations similar to the ones used in the studies described above.

4. Discussion

AD is one of the most common neurodegenerative diseases that affects millions of people worldwide and, due to its social economic costs, the search for a targeted and effective therapy remains a huge problem to solve [8,28]. The diagnosis and/or therapy of AD and also of many other neurological diseases is severely limited by the presence of the blood–brain barrier (BBB). Currently, nanoparticles seem to provide an important tool to overcome this problem, and within this context, nanoliposomes (decorated with OX-26 [anti-TIR MAb]) with increased targeting potential for the BBB [16,29] have been recently constructed. Also Aβ-MAb-decorated liposomes that showed high binding affinity toward Aβ peptide species [14] were recently developed. In line with the beneficial results obtained by several groups when using dually decorated nanoparticles for drug delivery purposes [6,7,30,31], dd-LIPs combining both previously used MBAs on the same vesicle were constructed, with the aim to generate a potential theranostic (drug and diagnostic imaging agent) carrier for AD. As a first step, the stability and integrity of the constructed LIPs were investigated as well as their interaction with human brain endothelial cells.

The experiments carried out herein reveal that the dd-LIPs can be used as a targeted therapeutic system, since they have nano-sized dimensions (Table 1) and adequate integrity during incubation in presence of serum proteins (Fig. 2). Their uptake by hCMEC/D3 cells is similar with that of immunoliposomes decorated only with OX-26, a result that is interesting, since it was previously observed [16] that when IgG was added as a second antibody on the same vesicle, uptake of OX-26 LIPs was significantly decreased compared to that of liposomes with only OX-26 on their surface, and it was suggested that the affinity of OX-26 for the receptors is affected by the presence of the second antibody. Thereby, it may be hypothesized that either the presence of Aβ-MAb as a second antibody does not alter the targeting potential of OX-26 (as previously observed with IgG) or that any such effect is complemented by independent additional interactions induced by the second antibody. In fact, it was demonstrated that the uptake of Aβ-MAb-LIPs by the cells is significantly higher (compared to that of corresponding control liposomes), suggesting that a specific interaction is taking place. Furthermore, the uptake values increased by 2–2.5 times, when incubations were carried out in the presence of Aβ1-42 peptides, suggesting that the peptide-LIP complex is transported through the hCMEC/D3 cell monolayer by the transporter of Aβ1-42 peptides. It is known [32] that the transporter which is primarily responsible for the blood-to-brain transcytosis of Aβ is RAGE. In fact, it was previously demonstrated that the BBB penetration of a radioiodine-labeled monoclonal antibody–Aβ immune complex was significantly enhanced relative to the antibody alone [33], and the authors implicated a saturable receptor-mediated mode of transport for the immune complex; similar results were reported earlier for apolipoprotein E [34].

The involvement of the RAGE receptor in the uptake and transcytosis of Aβ-MAb-LIPs by the BBB model used herein was proven after blocking RAGE with a specific RAGE MAb (Fig. 8). In good correlation with the current results, chitosan nanoparticles coated with anti-amyloid antibody were previously demonstrated to be taken up and transcytosed (through a polarized monolayer of bovine brain microvascular endothelial cells (BBMECs)) at double amounts compared with control nanoparticles. The later chitosan nanoparticles were also demonstrated to accumulate in various brain regions at 8–11 times higher amounts (compared to control NPs) following iv administration in wt mice [35], and the increased accumulation of the anti-amyloid antibody coated NPs in the brain was attributed to the antibody coating. Also, phospholipid nanoparticles on which the same (as above) MAb against fibrilar human amyloid-β42 was conjugated were demonstrated to bind to cerebral amyloid angiopathy (CAA) deposits in arterioles of AD transgenic mice after infusion into the external carotid artery [15].

In the current study, it was realized that components of FCS are essential for the interaction between hCMEC/D3 cells and Aβ-MAb-LIP (since the uptake of immunoliposomes was decreased to the level of control liposomes, when studied in buffer), in agreement with the fact that Ab is normally bound to albumin and/or specific lipoproteins in human plasma [25]. The same requirement applied for the Aβ1-42 peptide-induced enhanced uptake of Aβ-MAb-LIP (by cells) to occur (i.e. this was also demonstrated only in presence of cell culture medium including 10% FCS), implying that uptake of Aβ-MAb-LIP and Aβ1-42 peptide – Aβ-MAb-LIP complex is – at least to a certain degree – taking place through the same pathway, a fact that was further verified by blocking RAGE with RAGE MAb (Fig. 8).

The fact that the dd-LIP constructed herein are taken up by the brain cells at increased amounts in the presence of Aβ peptides suggests that these nanosystems may be ideal for AD therapy and/or diagnosis, where increased levels of the peptides are expected in AD-patient blood. Nevertheless, it has to be emphasized that the current nanosystems could be further optimized, by applying specific antibody attachment methodologies which will guarantee that the conjugation is situated at the constant region of the antibodies (which would allow the antibodies to work better since the antigen binding site will be free).

In the case that Aβ-MAb-LIPs are constructed for use as therapeutics in line with the peripheral sink hypothesis [36,37], any increase in the apical-to-basolateral transport of MBAs and/or MAb-LIPs may be negative, since it may enhance immunogenic effects and toxicity. Thereby, it is essential that when developing nanoparticulate systems for brain targeting or for use as Aβ-peptide
blood extractors (in line with the sink hypothesis), an emphasis is given on the type of the anti-amyloid antibody used, in relevance with previous findings about the importance of the antibody type [27,38,39]. Indeed, it has been recently proven [27] that when the C-terminal antibody is bound to Aβ, the complex (with an occupied Aβ N-terminal) is still recognized by RAGE and escorted into the brain. Conversely, when antibodies occupy the N-terminal or C-terminal antibody is bound to Aβ, the complex (with an unoccupied Aβ N-terminal) is still recognized by RAGE and escorted into the brain. Accordingly, when antibodies occupy the N-terminal or C-terminal antibody is bound to Aβ, the complex (with an unoccupied Aβ N-terminal) is still recognized by RAGE and escorted into the brain. Consequently, when antibodies occupy the N-terminal or C-terminal antibody is bound to Aβ, the complex (with an unoccupied Aβ N-terminal) is still recognized by RAGE and escorted into the brain.

5. Conclusions

Dual targeted liposomes decorated with antibodies to target the transferrin receptor (BBB) and the amyloid peptides, were developed. Results show that these dual targeted liposomes may have potential as carriers for delivery of drugs and/or imaging agents to the brain for treatment of AD.

It has been verified that liposomes that are decorated with Aβ-MAb are transcytosed across hCMEC/D3 cell monolayers by the RAGE receptor. This finding expands the applicability of this cellular model of BBB.

Acknowledgements

The research leading to these results has received funding from the European Community’s Seventh Framework Programme (FP7/ 2007-2013) under Grant agreement no. 212043.

References


