Functionalization of liposomes with ApoE-derived peptides at different density affects cellular uptake and drug transport across a blood-brain barrier model

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Abstract

A promising strategy to enhance blood-brain barrier penetration by drugs is the functionalization of nanocarriers with uptake-facilitating ligands. We studied the cellular uptake, by cultured RBE4 brain capillary endothelial cells, of nanoliposomes (NLs) covalently coupled with monomer or tandem dimer of apolipoprotein E (ApoE)-derived peptides (residues 141-150), at various densities. NLs without functionalization did not show either relevant membrane accumulation or cellular uptake, as monitored by confocal microscopy and quantified by fluorescence-activated cell sorting. Functionalization with peptides mediated an efficient NLs uptake that increased with peptide density; NLs carrying monomeric peptide performed the best. Moreover, we studied the ability of ApoE-NLs to enhance the transport of a drug payload through a RBE4 cell monolayer. The permeability of a tritiated curcumin derivative was enhanced after its entrapment into ApoE-NLs, in particular those functionalized with the dimer (+83% with respect to free drug, P < 0.01). Thus, these NLs appear particularly suitable for implementing further strategies for drug brain targeting.

From the Clinical Editor: Re and her collaborators present a method for delivering nanoliposomes via the blood brain barrier by utilizing peptide fragments including monomers or tandem dimers ApoE. This method may enable enhanced nanoliposome associated drug delivery via the blood-brain barrier, which would have enormous significance in neurodegenerative and other CNS disorders.

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Key words: Nanoliposomes; ApoE peptide; Brain endothelial cells; Blood-brain barrier

The brain is constantly confronted with the dilemma of protection from noxious substances from the blood vs. the delivery of vital metabolites. Endothelial cells, forming together with other cells the blood-brain barrier (BBB), are known as the “gatekeepers” of this trafficking. On the one hand, the

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.

1549-9634/$ – see front matter © 2011 Published by Elsevier Inc.
doi:10.1016/j.nano.2011.05.004

Please cite this article as: F., Re, et al, Functionalization of liposomes with ApoE-derived peptides at different density affects cellular uptake and drug transport across a... Nanomedicine: NBM 2011;7:551-559, doi:10.1016/j.nano.2011.05.004
that LDLr are present on capillary endothelial cells of several species and that their expression is upregulated in the BBB with respect to other endothelia. A further theoretical support comes from the observation that, in analogy with low-density lipoproteins, nanoparticles interacting with the LDLr via a specific apolipoprotein E (ApoE) amino acid sequence could be transported across the BBB by transcytosis, bypassing the lysosomal degradation.

Several studies with synthetic peptides have investigated the structural features of the LDLr-binding sequence of ApoE. It has been reported that the tandem dimer (141-155) is recognized by the LDLr, in contrast to the monomeric peptide (141-155). Also, a shorter sequence of this peptide, the tandem dimer (141-150), retains this ability. Probably as a consequence of this fact, sterically stabilized liposomes functionalized with ApoE tandem dimer (141-150) are efficiently taken up by rat brain capillary endothelial cells. Apart from the amino acid sequence, other factors may affect the cellular uptake of these peptides. These factors include the type of peptide, its mode of exposure to the cell surface, the nature of the cargo, and the chemical linkage between the peptide and the cargo. Other questions concerning the functionalization of nanoparticles with ApoE peptides are still not answered. For instance, the density of ApoE peptides may affect the cellular uptake of nanoparticles by BBB cells. The functionalization may enhance the BBB crossing of a trapped drug. The present study was undertaken to investigate these issues. For these purposes, the peptide sequence 141-150 of ApoE and its tandem dimer (141-150) were synthesized and utilized. As the vehicle, nanosized liposomes (nanoliposomes, NLs) were employed. Liposomes have attracted much attention as drug delivery systems and are particularly suitable tools for transport of peptide-decorated NLs or grown as a monolayer on a transwell system to investigate the transendothelial transport of peptide-decorated NLs or grown as a monolayer on a transwell system.

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-PhoEth) were purchased from Avanti Polar Lipids (Alabaster, Alabama). N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a diaza-s-indacene-3-dodecanoyl) sphingosyl phosphocholine (fluorescently labeled sphingomyelin, BODIPY-Sm) was from Molecular Probes, Invitrogen Srl (Milano, Italy). Polycarbonate filters for extrusion procedure were purchased from Millipore (Bedford, Massachusetts). The extruder was from Lipex Biomembranes (Vancouver, British Columbia, Canada). PD-10 columns were purchased from GE Healthcare (Uppsala, Sweden). All the media and supplements for cell cultures and Phalloidin were supplied by Invitrogen Srl (Milano, Italy). Triton X-100 was from Sigma-Aldrich.

Methods

Materials

Starting from native curcumin, an alkyne derivative (curcumin3, Figure 1) was synthesized according to the procedure described by Airoldi et al (unpublished data). The labeling of compound 3 was obtained using the method reported by La Ferla et al, and the [3H]curcumin4 was obtained. The synthesis procedure is reported in Figure 1.

Preparation of NLs

NLs were composed of Sm/Chol (1:1 molar ratio) mixed with 2.5 mol% of mal-PEG-PhoEth. For preparation of fluorescently labeled NLs, 0.5% (molar) of total Sm was substituted with BODIPY-Sm. Lipids were mixed in chloroform/methanol (2:1, vol/vol) and dried under a gentle stream of nitrogen followed by a vacuum pump for 3 hours 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. NLs were separated from possible unincorporated material by size-exclusion chromatography using PD-10 column and PBS as the eluent.
Preparation and characterization of NLs functionalized with ApoE peptide

mApoE or dApoE peptide was added to the NL dispersion in PBS to give a final peptide-to-mal-PEG-PhoEth molar ratio of 1:5 or 1.2:1 in the incubation mixture, and incubated overnight at room temperature (22–24°C) to form a thioether bond with mal-PEG-PhoEth. Peptide-bound NLs were separated from unbound peptide using a PD-10 column. The yield of coupling of the peptide to NLs was assessed as:

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\text{FI (nanoliposome fraction from PD10 column)} \times 100 \quad \text{FI (incubation mixture)}
\]

where FI is the fluorescence intensity (λ_ex = 280 nm; λ_em = 350 nm) measured using a Cary Eclipse spectrofluorimeter (Varian Inc., Palo Alto, California). The amount of peptide was calculated from the FI, using known amounts of peptide dissolved in buffer as standard. To estimate the peptide density on NL surfaces, the amount of peptide in the NL fraction was normalized to the lipid content, assayed as described below.

Preparation and characterization of NLs incorporating [3H]curcumin4

[3H]curcumin4 was added together with lipids in chloroform/methanol (2:1, vol/vol) during the initial phase of NL preparation, as described above. NLs incorporating [3H]curcumin4 were separated from unincorporated [3H]curcumin4 with a PD-10 column. The amount of [3H]curcumin4 incorporated into NLs was determined measuring the radioactivity by scintillation counting.

Physicochemical characterization of NLs

Lipid recovery after extrusion was evaluated by assaying the individual components: phospholipids were determined by phosphorus assay; cholesterol was determined as described. BODIPY-Sm recovery was assessed from the fluorescence emission intensity before and after extrusion. NL 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. NL 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. Standard deviations were calculated from at least three measurements. Stability was measured in buffer by following size and polydispersity index by dynamic light scattering for 3 days.

Culture of rat brain capillary endothelial cells

Rat brain endothelial cell line25 was provided as a gift by Dr. M. Aschner (Department of Pediatrics, Vanderbilt Kennedy Center, Nashville, Tennessee). Cells at passages 15–60 were grown on tissue culture flasks coated with type I collagen, in the following medium: Ham’s F10 Nutrient Mix and αMEM (1:1 vol/vol) supplemented with 10% (wt/vol) fetal bovine serum, penicillin-streptomycin 1% (wt/vol), L-glutamine 1% (wt/vol), and Geneticin (Invitrogen) 0.6% (300 μg/mL).

For uptake studies by confocal microscopy, RBE4 cells were seeded at a density of 32,000 cells/cm² on 25-mm glass coverslips precoated with collagen; confluent RBE4 monolayers were obtained typically by day 2. For uptake studies of [3H]curcumin4, 20,000 cells/cm² were cultured on type I collagen-coated 35-mm Petri dishes; confluent RBE4 monolayers were obtained typically by day 3. For flow cytometry, 32,000 cells/cm² were cultured on type I collagen-coated 35-mm Petri dishes. Subconfluent RBE4 monolayers were obtained typically by day 2.

Cell viability

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is taken up by cells and transformed into formazan by mitochondrial succinate dehydrogenase. Accumulation of formazan directly reflects the activity of mitochondria, an indirect measurement of cell viability. MTT stock solution (5 mg/mL) was added to each culture at a final concentration of 1.2 mM, and...
cells were incubated with NLs at 37°C, at a final lipid concentration of 100 μM for up to 48 hours, corresponding to twice the cell-doubling time. After removing MTT solution, the reaction was stopped by adding ethanol. Resuspended cells were centrifuged 10 minutes at 800 g. The absorbance was measured with a spectrophotometer at wavelength of 560 nm and at reference wavelength of 690 nm.

Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) was employed to estimate the uptake and the intracellular localization of the fluorescently labeled NLs. CLSM pictures were taken using an LSM710 inverted confocal laser scanning microscope equipped with a Plan-Neofluar 63×/1.4 oil objective (Carl Zeiss, Oberkochen, Germany). Excitation was performed using two ultraviolet-visible-laser diode 25 mV (405–488) and Ar-laser (540 nm) at 10% intensity. The pinhole was set to 1A. Image acquisition was done sequentially to minimize cross-talk between the fluorophores.

The cells were incubated at 37°C with NLs for 3 hours, rinsed three times with PBS, and fixed with a 10% (vol/vol) formalin solution. After three washes with PBS, cells were permeabilized with 0.2% (vol/vol) Triton X-100 in PBS for 15 minutes, then rinsed twice and incubated with a solution of 1% (vol/vol) Phalloidin (actin filaments staining) in PBS for 1 hour, then with 20 μM DAPI (nuclear staining) in PBS for 10 minutes. After three washes, the samples were mounted using polyvinyl alcohol mounting medium (Sigma-Aldrich). All washes were done with PBS.

Flow cytometry

RBE4 cells were incubated with NLs labeled with BODIPY-Sm at a final lipid concentration of 0.1 mM up to 3 hours at 37°C in serum-free medium. The cells were then washed with ice-cold PBS, treated with trypsin on ice, and collected with PBS supplemented with 10% of fetal bovine serum. Data corresponding to 30,000 events in a user-determined area were acquired for every experimental condition. The analysis was performed using DIVA software on a FACSCanto I (BD Biosciences, San Jose, California).

Experiments with [3H]curcumin4 incorporated in NLs

RBE4 cells were incubated with NLs incorporating about 20,000 dpm/mL of [3H]curcumin4, at a final total lipid concentration of 0.1 mM in PBS. Cells were also incubated with comparable radioactivity amounts of [3H]curcumin4 alone (not incorporated into NLs) for comparison. After 3 hours cells were washed, treated with trypsin, and then collected in PBS. Radioactivity of the cell suspension was measured by liquid scintillation counting.

For transport experiments across a cell monolayer, RBE4 cells were seeded in 12-well transwell inserts coated with type I collagen. Then 0.5 mL of cell suspensions containing 2.0 · 10^5 cells were added to the upper (donor) chamber, which was inserted into the lower (acceptor) chamber containing 1.0 mL of the culture medium. 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 Endohm chamber (World Precision Instruments, Sarasota, Florida) was obtained (typically between days 6 and 8). Wells were used when TEER value was higher than 40 Ω·cm^2.

Transendothelial permeability (PE) was calculated as previously described. All the permeability experiments were performed in serum-free medium at 37°C, adding 0.1 mM (as lipids) NLs incorporating about 20,000 dpm/mL of [3H] curcumin4 into the upper chamber. After adding the test substance to the upper compartment, samples were taken from the lower compartment at different times (0, 60, 180 minutes) for liquid scintillation counting. 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.

Statistical analysis

Each experiment was performed at least in triplicate. The differences were evaluated for statistical significance using Student’s t-test.

Results

NL characterization

The total lipid recovery of NLs was about 90%. The different lipid components of the mixtures were recovered with equal efficiency and always reflected the proportion in the starting mixture (data not shown). Final preparations of NLs were monodisperse (Table 1, A), with a mean size of 132 ± 10 nm. Zeta potentials of NLs are also reported in Table 1, A. As a further characterization of NLs, their size remained constant, within the experimental error, for up to 72 hours (Table 1, B).

ApoE-NL coupling

The tryptophan fluorescence spectrum of mApoE or dApoE, before and after incubation with mal-PEG-PhoEth NLs, showed a blue shift of the emission maximum from 354 nm to 350 nm (Figure 2, A), suggesting the coupling of the peptide with the NL maleimide. The yield of coupling was between 60 and 70%. After functionalization with ApoE peptides the size of NLs, reported in Table 1, A together with zeta potentials, slightly increased. The size of the peptide-NLs remained unchanged for at least 72 hours (Table 1, B). On increasing the molar ratio between peptide and mal-PEG-PhoEth (from 1.5 to 1.2:1), the density of both mApoE and dApoE peptides exposed on the NL surface increased (Figure 2, B). Assuming that 70,000 lipid molecules are in the outer layer of a NL with a diameter of 140 nm and containing 2.5 mol% of reactive mal-PEG-PhoEth, and a coupling efficiency of 70%, the densities after incubation at 1.2:1 or 1:5 peptide/mal-PEG-PhoEth molar ratio are ~1200 (high}
of fluorescence associated with the cells increased, on increasing sis, basically confirming the previous CLSM results. The amount evaluated by fluorescence-activated cell sorting (FACS) analy-

recycling regions (Figure 3, green spots below the plasma membrane and near the perinuclear (Figure 3, associated with cells increased with the density of the peptide studies), the amount of green (BODIPY-Sm) fluorescence (Figure 3, displayed neither membrane accumulation nor cellular uptake of fluorescence (Figure 3, mediated an efficient fluorescence uptake.

The uptake of fluorescently labeled NLs was quantitatively NLs functionalized with mApoE or dApoE, at LD or HD, displayed some cytotoxicity, depending on their lipid composition and even higher (13.74 ± 0.53%, P < 0.001) with mApoE-NLs at high peptide density, with respect to free [3H]curcumin4. (B) The size of NLs was measured at 0, 24, 48, and 72 hours.

density, HD) or 400 (low density, LD) peptide molecules per single NL particle, respectively.

Cell toxicity

NLs and ApoE-NLs were tested using the MTT assay. Up to 48 hours of incubation with the cells, all the NL preparations used were nontoxic, at least at the concentrations herein utilized (data not shown).

Cellular uptake of fluorescent NLs

Cellular uptake of fluorescence-labeled NLs was qualitatively evaluated by CLSM. NLs without surface functionalization displayed neither membrane accumulation nor cellular uptake of fluorescence (Figure 3, A). In contrast, the coupling with peptides, in particular with mApoE peptide (Figure 3, E and G), mediated an efficient fluorescence uptake.

NLs functionalized with mApoE or dApoE, at LD or HD, were studied. After 3 hours’ incubation with mApoE-NLs (the same maximum incubation time confirmed in our permeability studies), the amount of green (BODIPY-Sm) fluorescence associated with cells increased with the density of the peptide (Figure 3, C and E). The images showed the presence of hot green spots below the plasma membrane and near the perinuclear recycling regions (Figure 3, G, arrowhead). With dApoE-NLs, a smaller amount of fluorescence was associated with RBE4 cells at both low and high peptide density (Figure 3, B and D). In comparison with mApoE-NLs, fewer green spots were visible near the nucleus, although a clearly green signal was present just below the plasma membrane (Figure 3, F, arrow).

The uptake of fluorescently labeled NLs was quantitatively evaluated by fluorescence-activated cell sorting (FACS) analysis, basically confirming the previous CLSM results. The amount of fluorescence associated with the cells increased, on increasing the density of both mApoE and dApoE (Figure 4). mApoE-NLs, at high peptide density, showed the largest amount of fluorescence associated with cells.

Studies with [3H]curcumin4-NLs

The specific radioactivity of synthesized [3H]curcumin4 was 0.88 μCi/mg. For the uptake and permeability experiments we prepared NLs loading about 20,000 dpm of [3H]curcumin4 per 0.1 μmol lipids.

Uptake experiments

Upon cell incubation for 3 hours with free [3H]curcumin4, very small amounts of radioactivity (<2% of the administered dose) were found associated with cells. When [3H]curcumin4 was entrapped in ApoE-NLs, a strong enhancement of the cell-associated radioactivity was noted. In fact, when [3H]curcumin4 was entrapped within dApoE-NLs at high peptide density, the cell-associated radioactivity was higher (8.68 ± 0.36%, P < 0.001), and even higher (13.74 ± 0.53%, P < 0.001) with mApoE-NLs at high peptide density, with respect to free [3H]curcumin4.

Permeability experiments

After 7 days in vitro, TEER values were constant in relation to time (typically 45.1 ± 3.8 Ω•cm²). At this time, the PE value for sucrose was in the order of 0.87 ± 0.03 × 10⁻³ cm/min, similar to values reported previously (0.72 × 10⁻³ cm/min). Accordingly, all subsequent permeability experiments were performed after 7 days in vitro. After incubation with any NL formulation, the permeability of [¹⁴C]sucrose and TEER values did not change, within the experimental error (<3% and <1.5%, respectively), thus suggesting no adverse effect on cell monolayer integrity. The PE value of free [3H]curcumin4 (1.7 ± 0.03 × 10⁻³ cm²/min) was enhanced when the drug was incorporated in ApoE-NLs. In fact, after incorporation of [³H]curcumin4 into mApo-NLs or dApo-NLs, both at LD, a small increase of PE (+16.8% and +27.3%, respectively) was detected; however, a stronger enhancement (PE = 2.38 ± 0.36 × 10⁻³ cm²/min, +38.3%, P < 0.05) was detected after incorporation into mApoE-NLs at HD, and even stronger (3.15 ± 0.35 × 10⁻³ cm²/min, +83.1%, P < 0.01) after incorporation into dApoE-NLs at HD (Figure 5).

Discussion

This study aimed at the preparation and characterization of nanosized liposomal drug carriers, specifically functionalized to interact with endothelial BBB cells and with the potential ability to deliver drugs across the BBB. Development of strategies to deliver drugs to the central nervous system is of high importance, because many drug candidates are not able to permeate the BBB. Several nanocarrier systems have been proposed to overcome this problem. Among the various carriers introduced thus far, liposomes have such documented advantages as increased drug-loading capacity, versatile structural characteristics that permit easy surface decoration, biodegradability, biocompatibility, and minimal toxicity.

However, it should be pointed out that liposomes may also display some cytotoxicity, depending on their lipid composition and on their size. For instance, cationic liposomes are more
toxic than neutral and anionic ones, and small-sized liposomes have been reported to be more toxic than larger ones. Starting from these premises, we decided to use NLs composed of a matrix of sphingomyelin and cholesterol, because these agents have been repeatedly utilized in vivo for therapeutic purposes, displaying good circulation times in blood, biocompatibility, resistance to hydrolysis, and low ion permeability. However, because our investigation aims to formulate liposomes enhancing...
drug delivery to the brain, their potential neurotoxicity, reported for liposomes with a different lipid composition, should be taken into account. Future experiments will be devoted to investigate this important issue.

Recently, the focus of brain drug delivery has switched to mechanisms that use the endogenous capillary receptors, promoting membrane passage of specific large molecules by receptor-mediated transcytosis. Starting from the information that LDL receptors are upregulated at the BBB region with respect to other endothelia, within the present investigation we functionalized sphingomyelin-cholesterol NLs with ApoE-derived peptides (141-150 monomer and its tandem dimer) at different surface densities, so as to study their cell association, internalization, and the ability to deliver a drug across an in vitro BBB model, by means of fluorescence and radiochemical assays.

The investigation has utilized RBE4 cultured cells, a well-characterized model of rat brain capillary endothelial cells. NL association with RBE4 cells was quantified by means of FACS analysis; a substantial increase in cell-associated fluorescence was found in ApoE-NLs compared with non-targeted NLs. NL internalization within RBE4 cells was assessed by confocal fluorescence microscopy, showing that a substantial amount of ApoE-NLs are internalized by RBE4 cells.

Comparing the ability of NLs functionalized with ApoE peptides to interact with BBB endothelial cells, we found that either the nature of the peptide (monomer or dimer) or its density on the NL surface affect their cellular uptake. In fact, as opposed to non-functionalized NLs, mApoE or dApoE promote cells’ liposome uptake, though with different features: (1) on increasing the peptide density, the uptake increases for all ApoE-NLs; (2) the uptake of mApoE-NLs is higher than dApoE-NLs, both at low or high peptide density.

Although the cellular uptake of liposomes functionalized with dApoE has been previously investigated, the comparison with mApoE-NLs and the effect of peptide density has to our knowledge been reported within the present investigation for the first time.

The differences here reported between mApo- and dApo-NLs concerning their cellular uptake might be linked to their intrinsic mechanism of interaction with membranes. Because it has been reported that the tandem dimer (141-150) peptide binds to LDLR, we can speculate that our dApoE-NLs also retain this ability. On the other hand, although no information is available concerning the monomer 141-150, we can speculate that our mApoE-NL internalization is LDLr-independent, in analogy with the behavior of peptide 141-155. The understanding of the actual pathways involved in the uptake and subsequent intracellular fate of these cargos, even if it is outside the scope of the present investigation, is very important and deserves further investigation.

The ApoE-NL uptake by BBB cells might provide the first step of a transcellular transport of their drug payload. This hypothesis was tested on an in vitro system displaying characteristics and functionality mimicking the basic features of the BBB. We chose curcumin as our model drug because of its putative therapeutic properties in neurodegenerative diseases, utilizing a synthetic tritiated pyrazole derivative, [3H]curcumin, shown to improve the stability of the natural compound, while maintaining its binding features toward amyloid beta peptide.

Curcumin (diferuloylmethane) is a polyphenolic phytochemical derived from the rhizome of Curcuma species (family Zingiberaceae). It has been under intense scrutiny over the past...
two decades for its potent antioxidant, anti-inflammatory, and cancer chemopreventive properties. In addition, recent studies indicate a role for curcumin as a potential anti-amyloid agent because of its ability to inhibit amyloid beta peptide oligomerization in vitro and to disrupt existing amyloid plaques in an Alzheimer’s disease transgenic mouse model. Thus, its increased availability at the neurovascular junction of the cerebral microvasculature forming the BBB will be particularly desirable to treat neurological disorders such as Alzheimer’s disease and cerebrovascular amyloidosis. However, because of its poor solubility in water and usual solubility in solvents, the majority of curcumin that is administered by oral and intraperitoneal routes is confined to the gastrointestinal tract.

Some investigators have suspended curcumin in an oil-based medium and administered it to animals by the parenteral route. However, the measured amounts of curcumin in brain tissues varied considerably between studies, still remaining low, and this is probably related to the difficulties in overcoming the brain capillary endothelial cells.

In our in vitro BBB model, free [3H]curcumin did not associate with cells upon incubation. The cell-associated radioactivity strongly increased when [3H]curcumin was entrapped within ApoE-NLs; the highest association was detected with mApoE-NLs at high peptide density.

Entrapment of [3H]curcumin into ApoE-NLs also enhanced the PE value of free [3H]curcumin. The highest PE value recorded with dApoE-NLs at HD suggests their superior ability to enhance the flux of the drug across the cellular monolayer. To the best of our knowledge, these results concerning NLs functionalized with ApoE peptides at different densities are reported here for the first time.

We can speculate that dApoE-NLs interaction with cells via LDLr binding, and their subsequent transcytosis, thus escaping lysosomal degradation, is responsible for the higher PE of [3H]curcumin. On the contrary, the interaction of mApoE-NLs with cells, LDLr-independent, may be responsible of the higher cellular drug uptake. Of course, the LDLr-mediated transcytosis of dApoE-NLs must be taken as a hypothesis whose assessment requires further investigation. Indeed, we cannot exclude that, following endocytosis of dApoE-NLs, the entrapped drug is released and undergoes further transportation across the cell monolayer by diffusion. Alternatively, we cannot exclude that mApoE-NLs act by lowering the [3H]curcumin efflux from the cell.

Taken together, our results suggest that NLs functionalized with dApoE-peptide at HD could serve as versatile “nanovehicles” to deliver [3H]curcumin across the BBB, by providing a sheltered hydrophobic microenvironment, decreasing drug degradation, and therefore increasing its brain bioavailability. The information here gathered, using an in vitro model system, could be considered as the starting working hypothesis for future studies in vivo.

Acknowledgments

We are grateful to Prof. Michael Aschner for kindly providing rat brain endothelial cell line RBE4.

References