Neurochemistry International 108 (2017) 60–65

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

# Multifunctional liposomes interact with Abeta in human biological fluids: Therapeutic implications for Alzheimer's disease

Elisa Conti, PhD [a, *, 2], Maria Gregori [b, 2], Isabella Radice [a], Fulvio Da Re [a, c], Denise Grana [a], Francesca Re [b], Elisa Salvati [b, 1], Massimo Masserini [b], Carlo Ferrarese [a, c], Chiara Paola Zoia [a, 2], Lucio Tremolizzo [a, c, 2]

[a] Laboratory of Neurobiology, School of Medicine and Surgery and Milan Center for Neuroscience, University of Milano-Bicocca, Monza, Italy
[b] School of Medicine and Surgery, University of Milano-Bicocca, Monza and Nanomedicine Center NANOMIB, Italy
[c] Neurology, San Gerardo Hospital, Monza, Italy

ARTICLE INFO

*Article history:*
Received 28 September 2016
Received in revised form
17 February 2017
Accepted 21 February 2017
Available online 24 February 2017

*Keywords:*
Alzheimer's disease
Beta amyloid
Liposomes
Sink effect

ABSTRACT

The accumulation of extracellular amyloid beta (Abeta42) both in brain and in cerebral vessels characterizes Alzheimer's disease (AD) pathogenesis. Recently, the possibility to functionalize nanoparticles (NPs) surface with Abeta42 binding molecules, making them suitable tools for reducing Abeta42 burden has been shown effective in models of AD.

Aim of this work consisted in proving that NPs might be effective in sequestering Abeta42 in biological fluids, such as CSF and plasma. This demonstration is extremely important considering that these Abeta42 pools are in continuum with the brain parenchyma with drainage of Abeta from interstitial brain tissue to blood vessel and plasma.

In this work, liposomes (LIP) were functionalized as previously shown in order to promote high-affinity Abeta binding, *i.e.*, either with, phosphatidic acid (PA), or a modified Apolipoprotein E-derived peptide (mApo), or with a curcumin derivative (TREG); Abeta42 levels were determined by ELISA in CSF and plasma samples.

mApo-PA-LIP (25 and 250 μM) mildly albeit significantly sequestered Abeta42 proteins in CSF samples obtained from healthy subjects ($p < 0.01$). Analogously a significant binding (~20%) of Abeta42 ($p < 0.001$) was demonstrated following exposure to all functionalized liposomes in plasma samples obtained from selected AD or Down's syndrome patients expressing high levels of Abeta42. The same results were obtained by quantifying Abeta42 content after removal of liposome-bound Abeta by using gel filtration chromatography or ultracentrifugation on a discontinuous sucrose density gradient.

In conclusion, we demonstrate that functionalized liposomes significantly sequester Abeta42 in human biological fluids. These data may be critical for future in vivo administration tests using NPs for promoting sink effect.



## 1. Introduction

Alzheimer's disease (AD) represents the most frequent cause of dementia in the elderly population. Key event in AD pathogenesis is the deposition and accumulation both in brain and in microvessels of beta amyloid (Abeta) at the extracellular level. This deposition eventually leads to synaptic failure and consequent neurodegeneration. Accumulation of the most fibrillogenic isoform, Abeta 1–42, may start in normal subjects at about 40 years of age and raises with advancing age, possibly due to a disequilibrium between the production and catabolism of this peptide. Abeta oligomers, more than fibrils, seem to be the direct cause of synaptic dysfunction (Walsh and Selkoe, 2007). In fact, only a weak correlation between severity of dementia and Abeta fibrils density was shown in AD patients. On the other hand, recent studies show a strong correlation between soluble Abeta oligomer levels and the extent of synaptic loss and the severity of cognitive decline (Sakono

* Corresponding author. Laboratory of Neurobiology, School of Medicine and Surgery, UNIMIB, U8 building, via Cadore, 48, 20900, Monza (MB), Italy.
*E-mail address:* elisa.conti@unimib.it (E. Conti).
[1] Present address: IFOM, the FIRC Institute for Molecular Oncology Foundation, Milano, Italy.
[2] These authors contributed equally to the work.

http://dx.doi.org/10.1016/j.neuint.2017.02.012
0197-0186/

and Zako, 2010). Currently available therapies do not seem to be able to modify this pathogenic mechanism but are largely considered as symptomatic drugs. The possibility to reduce the amount of cerebral Abeta burden represents a potential therapeutic strategy under investigation. Brain and peripheral soluble Abeta are in equilibrium across the blood brain barrier (BBB); several clearance systems are responsible for the removal of Abeta from the brain. Receptors as LDLR, α2M and RAGE are involved in Abeta bidirectional passage through the BBB. Moreover, also perivascular drainage pathways represent clearance mechanism (Tarasoff-Conway et al., 2015). It has been proposed that a reduction in peripheral Abeta may result in decreased soluble levels in the brain, thereby reducing the formation of plaques (Matsuoka et al., 2003).

In recent years, many efforts to halt or reverse disease progression by immunomodulation strategies have been employed. Both active and passive immunization against Abeta has been tried first on animal model than in clinical trials on AD patients with alternating results (Wisniewski and Goñi, 2015). De Mattos and colleagues (DeMattos et al., 2001) suggested that only a small number of anti-Abeta antibodies administered via passive immunization in a mouse model of AD entered the central nervous system (CNS) and the degradation and clearance of central Abeta is plausibly reached by "peripheral sink" effect. Despite immunization of animal models gave promising results, clinical trial on human subject have not yet shown a real clinical benefit on disease progression, revealing immune toxic effects in some cases (Wisniewski and Goñi, 2015). In particular, during immunotherapies, the risk to develop transient but detrimental effects as vasogenic edema and microhemorrhages, known as amyloid related imaging abnormalities (ARIA), has been demonstrated. Moreover, this risk is associated to the presence of ApoE4 genotype (Salloway et al., 2009).

More recent strategies rely on the use of nanoparticles (NPs) as innovative vehicles able to localize and directly interact with Abeta. The possibility to functionalize NPs surface with molecules able to bind Abeta allows, in fact, in prospective, to have available tools of enormous potential for attempting Abeta burden reduction via the sink effect.

Studies on animal models of AD demonstrated that liposomes functionalized for Abeta binding and BBB crossing were able to reduce both total brain insoluble and oligomeric Abeta42, resulting in amelioration of impaired cognition (Balducci et al., 2014). Moreover, liposomes functionalized only for Abeta binding induced a reduction in brain and plasma Abeta via peripheral sink effect in APP/PS1 transgenic mice (Ordóñez-Gutiérrez et al., 2015). Lastly, bifunctionalized liposomes induced an efflux of Abeta oligomers from the "brain" side to the blood compartment in an in vitro model of BBB (Mancini et al., 2016).

In this work we tested the capacity of liposomes functionalized with different molecules to sequester Abeta42 in human biological fluids, *i.e.*, both plasma and cerebrospinal fluid (CSF). This proof of concept, in fact, is critical for thinking to future therapeutic applications of NPs in AD patients.

## 2. Materials and methods

### 2.1. Recruited subjects

Following approval by the ethical committee of the S. Gerardo Hospital (Monza, Italy), 20 AD patients were recruited (mean age ± SD: 75.5 ± 5.5, sex: 10 M/10 F, MMSE mean ± SD 18 ± 5.1). AD specialists diagnosed probable AD according to the NINCDS-ADRDA criteria (McKhann et al., 1984). Brain imaging and an extensive neuropsychological test battery excluded alternative diagnoses. Blood samples (5 ml) were collected in $K_2$EDTA tubes (4.08 mM final concentration), after overnight (ON) fasting. Plasma was obtained by centrifugation (3700*g*, 20 min) and stored at −80 °C until assay. Five Down's syndrome (DS) patients were included in the study as well: they were recruited from the "Eugenio Medea" Institute,"Associazione La Nostra Famiglia" Bosisio Parini (Lecco, Italy) and characterized by trisomy 21 chromosomal analyses.

Cerebrospinal fluid (CSF) was obtained from 10 control subjects, using a 21-gauge needle and collected in 10-mL polypropylene tubes. Part of the CSF was used for routine analysis (including leukocyte and erythrocyte count, glucose and total protein concentration). The remaining CSF was aliquoted into new polypropylene tubes, and stored at −20 °C for 24 h and then at −80 °C until analysis.

### 2.2. Preparation and characterization of liposomes and Abeta42

Liposomes (Lipo) composed of sphingomyelin from bovine brain (Sm, Sigma-Aldrich), cholesterol (Chol, Sigma-Aldrich), (1:1 molar ratio) and functionalized with dimyristoylphosphatidic acid (PA, Sigma-Aldrich) or bi-functionalized with PA and the peptide CWGLRKLRKRLLR-$NH_2$ (MW 1698.18 g/mol, mApo) derived from the receptor-binding domain (a.a. residues 141–150) of human Apolipoprotein E, were prepared and characterized as previously described (Gobbi et al., 2010; Bana et al., 2014).

Briefly, lipids were resuspended in chloroform/methanol (2:1, v:v) and dried under gentle stream of nitrogen followed by a vacuum pump for 3 h to remove traces of organic solvent. The resulting lipidic film was resuspended with 10 mM phosphate-buffered saline (PBS), pH 7.4, and extruded 10 times at 40 °C through a 100-nm pore size polycarbonate filter (Millipore Corp., Bedford, MA) under 20 bar nitrogen pressure with an extruder (Lipex Biomembranes, Vancouver, Canada). Lipid recovery after extrusion was assessed by phosphorous assay using the method of Stewart, (1980). mApo was added to maleimide containing liposomes in PBS to give a final peptide-to-maleimide molar ratio of 1.2:1. The mixture was incubated overnight at 25 °C. Peptide-bound NL was separated from the unbound peptide using a PD-10 column (GE Healthcare, Uppsala, Sweden). The yield of coupling and the amount of coupled peptide was assessed by tryptophan fluorescence intensity measurements, as reported (Re et al., 2011).

TREG liposomes were prepared and characterized as previously described (Sancini et al., 2013). They were composed of a matrix of Sm/Chol (1:1 molar ratio) mixed with 10 molar% of a PEGylated lipid containing an azido terminus (3-deoxy-1, 2-dipalmitoyl-3-(4′-methyl(O-(2-azidoethyl)- heptaethylenglycol-2-yl)-ethyl-carbamoylmethoxy ethylcarbamoyl-1H- 1′,2′,3′-triazol-1′-yl)-sn-glycerol) (Mourtas et al., 2011), for the coupling with TREG, a curcumin-derivative with a terminal alkyne group (*N*-propargyl 2-(3′, 5'-di(4-hydroxy-3-metoxystyryl)-1H-pyrazol-1′-yl)-acetamide) synthesized as previously described (Airoldi et al., 2011). For the coupling of TREG to liposomes, CuSO4 (8 mM), sodium ascorbate (145 mM) and TREG (100 mM in DMSO) were added to liposomes prepared as described above, and the reaction was stirred for 6 h, pH 6.5, at 25 °C. The resulting mixture was purified by gel filtration through a Sepharose 4B-CL column (Sigma-Aldrich, Milano, Italy).

All liposomes were characterized in terms of size and polydispersity using a ZetaPlus particle sizer (Brookhaven Instruments Corporation, Holtsville, NY, U.S.A.) at 25 °C and 0.25 mM total lipid concentration. Standard deviations were calculated from at least three measurements.

Lyophilized human Abeta42 peptide (Phoenix Pharmaceuticals) was solubilized in cold Tris–Buffer (pH 9) to obtain a 1 mg/ml solution of non-fibrillary Abeta42, as previously shown (Zoia et al., 2011).