Lipid-based nanoparticles with high binding affinity for amyloid-β_{1–42} peptide

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The neurotoxic beta-amyloid peptide (Aβ), formed in anomalous amounts in Alzheimer’s disease (AD), is released as monomer and then undergoes aggregation forming oligomers, fibrils and plaques in diseased brains. Aβ aggregates are considered as possible targets for therapy and/or diagnosis of AD. Since nanoparticles (NPs) are promising vehicles for imaging probes and therapeutic agents, we realized and characterized two types of NPs (liposomes and solid lipid nanoparticles, 145 and 76 nm average size, respectively) functionalized to target Aβ_{1–42} with high affinity. Preliminary immunostaining studies identified anionic phospholipids [phosphatidic acid (PA) and cardiolipin (CL)] as suitable Aβ_{1–42} ligands. PA/CL-functionalized, but not plain, NPs interacted with Aβ_{1–42} aggregates as indicated by ultracentrifugation experiments, in which binding reaction occurred in solution, and by Surface Plasmon Resonance (SPR) experiments, in which NPs flowed onto immobilized Aβ_{1–42}. All these experiments were carried out in buffered saline. SPR studies indicated that, when exposed on NPs surface, PA/CL display very high affinity for Aβ_{1–42} fibrils (22–60 nM), likely because of the occurrence of multivalent interactions which markedly decrease the dissociation of PA/CL NPs from Aβ. Noteworthy, PA/CL NPs did not bind to bovine serum albumin. The PA/CL NPs described in this work are endowed with the highest affinity for Aβ so far reported. These characteristics make our NPs a very promising vector for the targeted delivery of potential new diagnostic and therapeutic molecules to be tested in appropriate animal models.

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1. Introduction

Alzheimer’s disease (AD) is the most common neurodegenerative disorder in the elderly. AD causes continuous deterioration of higher nervous functions, due to progressive and irreversible degeneration of the limbic and other association cortices, leading to the total loss of autonomy and eventually to death. In the EU, about 5 million people have dementia, with AD accounting for over 3 million, and double of this figure is predicted by 2040 in Western Europe and treble in Eastern Europe. One in 20 people over 65, and one in five over 85, have AD; therefore, AD is considered as a major public health problem due to ageing of the population [1]. Although substantial progress has been made in the understanding of the molecular mechanisms underlying AD, there remains an urgent need to identify effective therapies and early detection strategies, in order to avert a financially overwhelming public health problem. One of the hallmarks of AD is the formation of anomalous amounts of β-amyloid (Aβ) a 40–42 residues-long peptide produced by abnormal proteolytic cleavage of the Amyloid Precursor Protein (APP) [2,3]. Aβ peptide is released from cells in a soluble form, and progressively undergoes aggregation forming oligomers, multimers and fibrils, and ending with deposition of extracellular plaques, one of the histopathological hallmarks of AD, detectable in diseased brain [4,5]. The predominant and initial species deposited in the brain parenchyma is Aβ_{1–42} [6]. Oligomers have been indicated as the most toxic Aβ species [7,8], likely appearing before plaque deposition in an early stage of AD pathology. However, protofibrillar and fibrillar aggregates of the peptide were also shown to be toxic [9–11]. Therefore, targeting of cerebral Aβ_{1–42} in all its aggregation forms has been suggested for therapeutic and/or diagnostic purposes of the disease [3,12–14]. Moreover, it has been
reported that brain and blood Aβ are in equilibrium, through the blood–brain barrier (BBB), and that peripheral sequestration of Aβ may shift this equilibrium towards the blood, eventually drawing out the excess from the brain ("sink" effect) [15]. Therefore, for the therapy of AD, also blood Aβ could be worth targeting.

Nanoparticles (NPs) are an attractive mean for these tasks, being suitable vehicles for imaging probes and therapeutic agents and for the possibility to functionalize their surface with target-specific ligands. Moreover, multivalent interactions may strikingly increase the affinity of functionalized NPs for their targets [16–18].

Within the present investigation we describe the preparation and characterization of NPs functionalized to target with high affinity Aβ(1–42) peptide in all its aggregation forms. In particular, we used lipid based nanoparticles liposomes or solid lipid nanoparticles (SLN), and focused our attention on amphipatic lipids as candidate specific ligands for Aβ(1–42). In fact, the involvement of membrane lipids in AD has been extensively studied, and a number of investigations reported their ability to interact with the peptide [19–23]. However, the majority of these studies was aimed to assess the influence of lipids upon Aβ(1–42) aggregation paradigm [24] whereas, to the best of our knowledge, the information about the specificity of binding are circumscribed to a few molecules, among which gangliosides display the highest affinity [25–27].

2. Materials and methods

2.1. Materials

Monosialogangliosides GM1, GM2 and GM3; disialogangliosides GD1α, GD1β and GD3; trisialoganglioside GT1b from bovine brain; sphingomyelin from bovine brain (Sm); cholesterol (Chol); dimystearylphosphatidic acid (PA); dihosphatidylglycerol (PG); cardiolipin (CL); 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC); phosphatidylethanolamine (PE)and Sephadex G75 were purchased from Sigma–Aldrich (Milano, Italy). Phospholipon 90G (purified soy lecithin with a phosphatidylcholine content of at least 94% as by supplier) was supplied from Phospholipon (Cologne, Germany), diestearylphosphatidic acid was purchased from NOF (Tokyo, Japan). Stearic acid was purchased from Merck (Darmstadt, Germany). Sodium taurocholate was purchased from PCA (Basaluzzo, Italy). [3H]Dipalmitoyl-phosphatidylcholine, ECL reagents and Sepharose CL-4B were from Amersham (Castle Hill, NSW, Australia). PVDF membrane was from Millipore. Recombinant human Aβ(1–42) HPF and DMSO were from Sigma–Aldrich (Milano, Italy); in addition, also Aβ(1–42) synthetized in our labs as described below was utilized. Mouse monoclonal Aβ(1–42) antibody mAb 6E10 was purchased from Signet (Dedham, MA). All other chemicals were reagent grade. Ultrapure and deionized water were obtained from Direct-Q system (Millipore, Italy).

2.2. Preparation of Aβ(1–42) in different aggregation forms

2.2.1. Monomers

Aβ(1–42) monomeric preparations were obtained from the commercial peptide or from the in-house produced peptide as previously described [28,29]. The commercial peptide was dissolved in HPF at 1 mg/ml concentration to monomerize pre-existing aggregates; HPF was then allowed to evaporate and the resulting peptide 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 obtain the peptide solution.

2.2.2. Oligomers

To obtain Aβ(1–42) oligomeric preparations, the monomeric peptide solutions (both the commercial peptide and the in-house produced peptide) were diluted to 100 μM in 50 mM phosphate buffer, 150 mM NaCl, pH 7.4, and incubated for 24 h at 4 °C [32]. The presence of oligomers in these preparations has been previously confirmed and characterized [30,31]. The oligomeric preparations of Aβ(1–42) peptide obtained with this procedure is here referred to as “oligomers”.

2.2.3. Fibris

To obtain Aβ(1–42) fibril preparations, the monomeric peptide solutions (both the commercial peptide and the in-house produced peptide) were diluted with water to 100 μM, acidified to pH 2.0 with HCl and left for 24 h at 37 °C [27,28]. Kinetic studies with circular dichroism and thioflavin-T clearly indicated that these conditions enable to reach the maximal level of β-sheets structures, whereas AFM directly confirmed the presence of fibrillar species [30,31]. The fibrillar preparations of Aβ(1–42) peptide obtained with this procedure are therefore referred here to as “fibrils”.

2.3. Screening of Aβ(1–42)/lipids interaction by TLC-immunostaining

Preliminary screening of Aβ(1–42)/lipid interaction was carried out following the procedure utilized for Cholera toxin B subunit and gangliosides [33] with small modifications. Briefly, equal amounts (1 nmole or 0.5 nmole) of different lipids (gangliosides, Sm, PC, PE, Chol, PG, PA and CL) were applied on a TLC plate and developed with a chloroform/methanol/CaCl2 0.25% (50:35.5:5 v/v) solvent. The TLC plate was incubated with polyisobutylmethacrylate in chloroform for 3 times. The dried plate was incubated in the blocking solution [0.1 M Tris, 0.14 M NaCl, 1% bovine serum albumin ([BSA]) at RT for 30 min. After washing with phosphate buffered saline (PBS), the plate was incubated with monomeric Aβ(1–42) (2.5 ng/ml) in 15 BSA in PBS) for 90 min at RT. After peptide incubation, the plate was incubated for 1 h at RT with the mAb 6E10 diluted in 1% BSA in PBS 1:1 (1000) and then for 45 min at RT with HRP-conjugated IgG anti-mouse in the same buffer (1:20000), followed by ECL detection. Spots were digitally semi-quantitatively estimated by a Kodak Image station 2000R software. Parallel, a TLC plate was also prepared in which the lipids, after the chromatographic run, were visualized by iodine vapor. The purpose of this plate was to compare the Rf of bands visualized by immunoblotting with those visualized by chemical staining of standard lipids.

2.4. Nanoparticles preparation

2.4.1. Liposomes

Liposomes were composed of a matrix of Sm/Chol (1:1 molar ratio) mixed or not with 5 or 20 molar % of one of the following lipids: gangliosides, Sm, PC, PE, Chol, PG, PA or CL. 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 residual solvent. The resulting lipid film was rehydrated in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, vortexed and then extruded 10 times at 40 °C through a stack of two polycarbonate filters (100–nm pore size diameter, Millipore Corp., Bedford, MA) under 20 bar nitrogen pressure with an extruder (Lipex Biomembranes, Vancouver, Canada). Liposomes were separated from possible unincorporated material by passage through a Sepharose CL–4B [27]. Lipid recovery after extrusion was assessed by assaying the individual components: phospholipid recovery was determined by phosphorous assay using the method of Bartlett [34]; glycolipid content was determined as lipid-bound sialic acid [35]. Cholesterol content was determined as described in Ref. [36]. In the case of mixtures containing different phospholipids, the recovery of the individual components after extrusion was assessed as follows: phospholipids were separated by HPTLC using the solvent system chloroform/methanol/acetic acid/water, 60:45:4:2 (v/v/v/v). The bands corresponding to each phospholipid were visualized with iodine, scraped off from the plate and submitted to phosphorous assay [34]. Identification of lipids after separation was assessed by co-migration with standard lipids loaded on the same plate.

The presence of an aqueous core, indicative of the ability to trap hydrophobic molecules, was established by using calcein using the procedure described in Ref. [37]. In some small modifications. For this purpose, the cholinergic solvent lipid film was carried out with buffer containing 60 mM calcein. To remove the unencapsulated calcein, the liposome suspension was passed through a Sephadex G75 column (25x1 cm) at 4 °C. Liposomes elution was assessed by DLS (Dynamic Light Scattering) and entrapment of calcein was assessed by dequenching of fluorescence (which is self-quenched at the high calcein concentrations inside liposomes) after the addition of Triton X-100.

In some instances, for studies of binding performed with ultracentrifugation, tritiated phosphatidylcholine was added as a tracer to follow lipid distribution by radioactivity counting.

2.4.2. Solid lipid nanoparticles (SLN)

SLN were prepared by oil-in-water (O/W) warm microemulsion method [38] using stearic acid (0.35 mM) as internal phase, phospholipon 90G as surfactant (0.070 mM) and ultrapure water as external phase (55.55 mM). Functionalized SLN were substituted substituting 5% mol of Phospholipon 90G with diestearylphosphatidic acid (PA). Stearic acid and phospholipon 90G were heated together at 70 °C then sodium taurocholate dissolved in ultrapure water, and heated at the same temperature, was added to obtain an optically clear system. The O/W microemulsion was dispersed 1:10 in cold ultrapure water maintained at 2–3 °C under mechanical stirring (700–900 rpm); the obtained SLN aqueous dispersion was washed three times by tangential filtration on Vivaflow 50 module (Sartorius Stedim Biotech, Germany) with a 100,000 MWCO RC membrane.

The preparation of functionalized SLN, 20% PA was introduced in previous formulation by substituting the same percentage of Phospholipon 90G. As
amphiphilic molecule, PA, is going to locate at interphase of microemulsion with the hydrophilic moiety (phosphate group) towards the aqueous phase and the lipophilic moiety (acyl chains) towards the oil phase, allowing the hydrophilic portion negatively charged to remain exposed on the surface of SLN after quenching of the microemulsion in cold water.

2.5. Characterization of nanoparticles

The size, polydispersity and ζ-potential of the liposome and SLN were determined using a ZetaPlus particle sizer and ζ-potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, U.S.A.). The size and polydispersity measurements were performed at 25 °C. Liposomes, prepared in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, were diluted at 0.25 mM total lipid concentration. SLN samples were diluted 1:100 with deionized water. The particle size was assessed by DLS with a 652 nm laser beam. Particle size and polydispersity index were obtained from the size distribution autocorrelation function of the light scattered at a fixed angle of 90°. The correlation function was analyzed by means of a two cumulant expansion. The ζ-potential was measured at 25 °C. Each measurement was performed on freshly prepared liposome samples diluted 1:25 with deionized water. The liposome measurements have been performed with ZetaPALMS device, which is basically an interferometric doppler velocimetry. The reported data are the mean of at least five measurements. The SLN ζ-potential was measured by laser doppler electrophoresis using a Zetasizer 3000HSA (Malvern Instruments, UK) with a capillary cell. 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 DLS for 48 h.

2.6. Binding of liposomes to Aβ1–42, investigated by Surface Plasma Resonance (SPR)

For these studies we used the Protein XPR36 (Biorad) apparatus, which has six parallel flow channels that can be used to uniformly immobilize strips of six “ligands” on the sensor surface. Aβ1–42 monomers, oligomers or fibrils were immobilized in parallel-flow channels of a GLC sensor chip (Biorad) using amine-coupling chemistry. Briefly, after surface activation the peptide solutions (10 μM in acetate buffer pH 4.0) were injected for 5 min at a flow rate of 30 μL/min, and the remaining activated groups were blocked with ethanolamine, pH 8.0. The final immobilization levels were similar, about 2500 Resonance Units (1 RU = 1 pg protein/mm²). Before performing experiments with liposomes, we previously checked that all the Aβ species immobilized were binding with high affinity the anti-Aβ antibody 6E10, and that only fibrils were binding the β-sheet specific ligand Congo-Red. In some cases, bovine serum albumin was immobilized in a parallel flow channel, as a reference protein. Another “reference” surface was always prepared in parallel using the same immobilization procedure but without addition of the peptide (empty surface). The fluidic system of Protein XPR36 can automatically rotate 90 ° so that up to six different analytes (e.g. different nanoparticles preparations, or different concentrations of the same nanoparticle) could be injected simultaneously over all the different immobilized molecules [39].

2.7. Binding of liposomes to Aβ1–42, investigated by ultra-centrifugation on a discontinuous sucrose density gradient

0.5 μM of Aβ1–42 monomers, oligomers or fibrils were incubated for different times with liposomes (incorporating or not PA, CL or GM1) at different concentrations (2.5 μM total lipids for monomers and oligomers; 2.5 μM and 0.5 μM total lipids for fibrils), containing a tritiated lipid tracer (less than 0.0001% of total lipids), in a final volume of 450 μL of 10 mM Tris, 150 mM NaCl, 1 mM EDTA pH 7.4 at 37 °C, under continuous agitation. After incubation the peptide bound to liposomes was separated from free peptide by flotation in a discontinuous sucrose density gradient performed as follows. Incubation mixtures of Aβ1–42 with liposomes, were mixed with 1350 μL 80% sucrose in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4, to obtain a final 60% sucrose concentration. On top of this suspension, 1350 μL of 50% sucrose in the same buffer and 1350 μL of sucrose-free buffer were layered in the order. The samples were centrifuged in a Beckman MLS 50 rotor at 140,000 × g for 2 h in polycarbonate tubes; 10 fractions of 450 μL each were collected from the top of the gradient and assayed for lipid and peptide content [40,41]. The distribution of lipids along the gradient was followed by phosphorous assay or by counting the liposome-associated radioactivity by liquid scintillation. The distribution of the peptide was followed by dot-blot procedure: briefly, 5 μL of each gradient fraction were spotted on a PVDF membrane (pre-wet in methanol), washed for 5 min in water, followed by TBS and left to air dry. Membrane was incubated for 90 min at RT with the mAb 6E10 diluted in non-fat milk in TBS 1: (1:1000) and then for 2 h at RT with HRP-conjugated anti-mouse in the same buffer (1:20000), followed by ECL detection. Chemiluminescent spots were digitally semi-quantitatively estimated by a Kodak Image station 2000R using Kodak Molecular Imaging Software Version 4.0.

Fractions of the gradient carrying both lipids and peptide (usually fractions 1–5) were considered as “liposome-bound peptide”, and fractions containing only peptide (usually fractions 6–10) were considered as “unbound peptide”. The proportion of peptide bound was expressed as the % ratio between the amount of peptide in the fractions 1–5 over the total peptide amount.

3. Results

3.1. Screening of Aβ1–42/lipids interaction by TLC-immunostaining

To collect some preliminary information on the interaction of Aβ1–42 with lipids, we utilized a TLC-immunostaining technique. Lipids under testing were separated by TLC and the plate was incubated with monomeric Aβ1–42, followed by anti-Aβ antibodies and ECL detection. When 0.5 nmol of each lipid were spotted on the plate, chemoluminescence was detected in correspondence of all the gangliosides, PA and CL, and in correspondence with gangliosides and PA using 0.1 nmol of each lipid (Fig. 1). No apparent association of peptide was detectable with PC, Sm, PE, Chol and PG, under these experimental conditions.

3.2. Characterization of NPs

3.2.1. Liposomes

Liposomes were prepared by extrusion procedure. After extrusion, either the total recovery of lipids or the recovery of each lipid component of the mixture were assessed. The total lipid recovery was about 90% for all the samples. The different lipid components of the mixtures were recovered with equal efficiency after the extrusion procedure and always reflected the proportion in the starting mixture. The samples used for binding experiments were assayed for lipid content before the experiments. The final preparations of liposomes were monodispersed (Table 1). ζ-Potential distributions of liposomal preparations were also measured and are reported in Table 1. Phospholipid composition predominantly influenced surface charge of liposomes, and, as expected, ζ-potential decreased with increasing concentrations of anionic gangliosides.

As a further characterization of the most relevant liposomes (see following results), we investigated the stability of liposomes containing PA or CL, following their physical features by DLS upon incubation in phosphate buffered saline for up to 48 h. Size remained constant, within the experimental error (Table 2). In additional experiments, the liposomes were prepared in the presence of calcine and submitted to column chromatography. Calcein fluorescence was detected also in the fractions coinciding with liposomes and increased after Triton treatment (data not shown), indicating its entrapment.

3.2.2. SLN

ζ-potential of functionalized SLN (Table 1) were more negative after substitution with PA of surfactant Phospholipon 90C, suggesting increasing percentage of incorporation of the anionic moiety of PA on the surface of SLN. Formulations with PA showed higher average diameter of SLN dispersion.

3.3. Binding of liposomes to Aβ1–42, investigated by Surface Plasma Resonance (SPR)

In the first SPR experimental session, we flowed liposomes of different compositions over an empty sensor surface, used as reference (Fig. 2A) and, in parallel over a sensor surface coated with Aβ1–42 fibrils (Fig. 2B). No or negligible binding was detected when flowing liposomes composed of Sm/Chol alone or embedding 20% PE, 20% PC or 5% PG, even at the highest concentration tested (1 mM total lipids), whereas some binding was observed with liposomes containing 5% GM1, confirming previous results [22].
A very marked binding response, specific for the surface coated with \( \text{A}_{\beta1-42} \) fibrils, was observed when flowing Sm/Chol liposomes embedding 20% (M) PA.

The binding of PA-liposomes to \( \text{A}_{\beta1-42} \) fibrils was concentration-dependent (Fig. 2C). The separate analysis of the three sensorgrams shown in Fig. 2C, according to the simplest equation modeling a Langmuir 1:1 interaction, resulted in \( K_d \) values in the submicromolar range (560–42 nM) mainly because of a very low dissociation rate constant (1–9 \( \times 10^{-5} \) s\(^{-1}\)). Assuming that the lipid components are equally distributed between the two leaflets of the liposome bilayer and that the interaction with \( \text{A}_{\beta1-42} \) is occurring only with the outer layer, then the \( K_d \) value of PA can be recalculated as 56–61 nm.

Importantly, however, the three sensorgrams could not be fitted together (global fitting), in particular because each sensorgram showed different estimates of the maximum binding attainable (\( R_{\text{max}} \)). The fact that \( R_{\text{max}} \) values are roughly proportional to the liposomal-PA concentrations (177, 436 and 1400 RU for 2.5, 7.5 and 25 \( \mu \)M PA) on A\(_{\beta1-42}\) fibrils, whereas no binding was observed on BSA or on the empty surface. “Plain” Sm/Chol liposomes, injected in parallel over the same four sensor surfaces, showed no or negligible binding (Fig. 3A). The involvement of PA for the specific recognition of A\(_{\beta1-42}\) fibrils was confirmed when injecting another type of nanoparticles, SLNs, with or without PA. In this case too, we found a specific binding of SLN-PA (2.6 \( \mu \)M PA) on A\(_{\beta1-42}\) fibrils but not on A\(_{\beta1-42}\) monomers or BSA (Fig. 3E).

Table 1

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<tr>
<th>Liposomes (Sm/Chol 1:1) and SLN containing or not CL, PA, PE, PG, PC or GM1 were characterized by Dynamic Light Scattering (see text for details) in order to determine size distribution and zeta-potential.</th>
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<tr>
<td>Av. diameter ± SD (nm)</td>
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<td>Liposomes</td>
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<td>Sm/Chol</td>
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<td>Solid lipid nanoparticles</td>
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<td>SLN/PA 5%</td>
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Table 2

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<tr>
<th>Sample</th>
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<th>Av. diameter ± SD (nm)</th>
<th>Av. Diameter ± SD (nm)</th>
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<tr>
<td>t = 0</td>
<td>144 ± 10</td>
<td>148 ± 11</td>
<td>145 ± 12</td>
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<td>t = 24 h</td>
<td>146 ± 12</td>
<td>142 ± 13</td>
<td>146 ± 10</td>
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<td>t = 48 h</td>
<td>151 ± 14</td>
<td>149 ± 12</td>
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SD – standard deviation; PC – phosphatidylcholine; Sm – sphingomyelin; PE – phosphatidylethanolamine; Chol – cholesterol; PA – phosphatidic acid; CL – cardiolipin; PE – phosphatidylethanolamine; Chol – cholesterol; PA – phosphatidic acid; CL – cardiolipin.
dissociation phase. Thus, about 55% of the bound SLNs dissociated quite rapidly ($9 \times 10^{-3} \text{s}^{-1}$, $K_d = 2.2 \mu M$), whereas the remaining 45% shows a pseudo-irreversible binding with a very slow dissociation rate ($\leq 7 \times 10^{-5} \text{s}^{-1}$, apparent $K_d$ of PA $\leq 17 \text{nm}$).

A third experimental session aimed at evaluating the binding properties of PA-liposomes on different Aβ$_{1-42}$ species: monomers, fibrils and also oligomers. In these experiments we also tested liposomes containing cardiolipin (CL). Liposomes contained PA or CL at different proportions (5% or 20%).

In Fig. 4 are reported the normalized sensorgrams indicating their specific binding to the different Aβ species. In all cases, the larger binding was found on Aβ$_{1-42}$ fibrils whereas much lower or no binding was found on Aβ$_{1-42}$ monomers. Intermediate binding levels were found on the surfaces with oligomers despite the fact that the actual amount of oligomers present in the preparation used for immobilization is 5–10%, the remaining being monomers [30]. The apparent $K_d$ values of the phospholipid in the external layer on the fibrils were 87 and 22 nm for PA (if present in liposomes at 5 or 20%, respectively), and 75 and 44 nm for CL (if present in liposomes at 5 or 20%, respectively).

3.4. Binding of liposomes to Aβ$_{1-42}$ fibrils investigated by ultracentrifugation on a discontinuous sucrose density gradient

Liposomes composed of Sm/Chol, embedding or not CL or PA or GM1 were incubated with monomeric, oligomeric or fibrillar preparations of Aβ$_{1-42}$ (0.5 μM) at 1:5 peptide: lipids molar ratio and, for fibrillar Aβ$_{1-42}$, also 1:1.

After incubation, the mixtures were submitted to ultracentrifugation on discontinuous sucrose density gradient for separation of bound and unbound peptide. Gradient fractions were collected and analyzed for the lipid and peptide content. When loaded alone on the gradient, lipids were recovered within the upper, low-density fractions, the peptide, when loaded alone, remained in the bottom fractions (Fig. 5A, C). Upon incubation, lipids and a variable proportion of the peptide, depending on the liposome samples and on the lipid:peptide incubation ratio, were recovered together in the low-density upper fractions of the gradient: an example is reported in Fig. 5B, D. In order to set up the incubation conditions, liposomes were added with Aβ$_{1-42}$ at 37 °C, and the amount of peptide bound was assessed by dot-blot at different times. For all the samples, after 15 min incubation the amount of liposome-bound peptide did not increase anymore: this time of incubation was utilized in the subsequent experiments (Fig. 5E).

Monomers. After incubation of monomeric Aβ with Sm/Chol liposomes containing or not PA, CL or GM1 at Aβ$_{1-42}$:total lipids = 1:5 molar ratio, a small but detectable binding of peptide was found only with liposomes containing 20 or 5% PA or CL (4–5% or 3–5% peptide bound, respectively) (Fig. 6A). No relevant binding was detected with Sm/Chol and Sm/Chol/GM1 liposomes.

Oligomers. After incubation of liposomes with oligomeric Aβ$_{1-42}$, a relevant binding was detected with liposomes containing 20% CL
(36% peptide bound) and with liposomes containing 20% PA (6% peptide bound) (Fig. 6B). No binding was displayed by liposomes containing 5% of PA or CL, or by Sm/Chol and Sm/Chol/GM1 liposomes.

Fibrils. After incubation of liposomes with fibrillar Aβ1-42 at 1:5 (Aβ1-42:total lipids) molar ratio, large amounts of Aβ (from about 50 to 90%) were bound to all liposomes with the exception of “plain” liposomes (Fig. 6C). When the total amount of lipids was further decreased (Aβ1-42:total lipids = 1:1 molar ratio), lesser amounts of Aβ (from about 5 to 18%) were bound to liposomes, the maximum displayed by Sm/Chol/CL 20%. Also in this case no detectable binding of fibrils to “plain” liposomes was recorded (Fig. 6D).

4. Discussion

In order to gather preliminary clues on the existence of specific interactions between amphipatic lipids and Aβ peptide, we performed a screening using an in vitro TLC-immunoassay, originally
described for gangliosides and Cholera toxin. The results suggested that, among the different lipids tested, PA, CL and gangliosides could be the best candidate ligands. Then, we utilized amphipatic lipids for the preparation of lipid-based nanoparticles, namely liposomes and SLN, able to target with high affinity peptide in all its aggregation forms.

Liposomes and SLN are biocompatible nanoscale materials that have been extensively studied in the recent years, showing interesting features, such as stealth characteristics (ability to avoid the reticulo-endothelial system), biocompatibility and high physical stability, supporting their use for therapy and diagnosis of human diseases. Moreover, and particularly useful in our case, the insertion of amphipatic lipids, beside those already present in their formulations, is easily carried out during the preparation of these NPs.

We utilized liposomes composed by a matrix of Sm and Chol, in equimolar ratio, for the following reasons: i) such liposomes embedding gangliosides have been utilized for assessing the affinity of these glycolipids for Ap1-42: the K<sub>d</sub> in this system proved to be in the micromolar range; ii) Sm/Chol liposomes have been repeatedly utilized in vivo for therapeutic purposes, displaying good circulation times in blood, biocompatibility, resistance to hydrolysis, low ion permeability; iii) Sm/Chol bilayers are known to form raft-like or liquid-ordered domains that are representative of a native cellular membrane where Ap1-42 accumulates; iv) the presence of cholesterol was shown to strengthen the Ap-membrane interaction. It should be pointed out that also other lipid matrices have been utilized for pharmacologic purposes and it will be interesting to test them in future experiments.

Within this investigation we performed some experiment also with SLN. Stearic acid has been chosen as the lipid matrix because of SLN of small size are obtained, and because of the few components required. This kind of formulation, with same excipients but loaded with drugs, showed improvement of drug intracellular accumulation in <i>in vitro</i> models, and showed to improve pharmacokinetic parameters in <i>in vivo</i> models. Same formulation as well has been previously tested for PEG surface functionalisation, obtaining good circulation time and BBB overcoming in animal model.

For determination of the affinity constants of binding between NPs and Ap1-42 we utilized the SPR technique, flowing NPs over immobilized peptide. This is a very suitable approach to characterize the binding properties of functionalized nanodevices, providing very useful information on the binding constants and the avidity effects (see below). All experiments were performed in a buffer containing a physiological concentration of salt (150 mM NaCl) and with a pH of 7.4 in order to mimic the biological fluids. Our SPR data indicated that “plain” Sm/Chol liposomes do not bind to immobilized Ap fibrils. The highest binding was found with liposomes containing the anionic phospholipids PA or CL, suggesting the involvement of electrostatic interactions. However, the very low binding displayed by liposomes containing PE, PC or PG, in spite of the presence of localized anionic charges on these lipids, calls also for the involvement of hydrophobic interactions. An intermediate binding was found with liposomes containing GM1 ganglioside, with estimated affinities in the micromolar range. This finding confirms previous data reported in literature, suggesting that non-electrostatic interactions are involved also in the

Fig. 4. CL- and PA-liposomes preferably bind to Ap1-42 fibrils and oligomers. Liposomes (Sm:Chol 1:1) containing 5% or 20% PA or CL (panel A and B = 5%; C and D = 20%) corresponding to a concentration of exposed PA or CL of 12.5 and 50 μM were flowed for 3 min (as indicated by the bars) simultaneously over a sensor surface previously coated with Ap1-42 monomers, oligomers or fibrils. All the panels show the specific binding of CL- and PA-liposomes to Ap1-42 species, obtained after subtraction of the binding of the same nanoparticles to the empty surface (non-specific binding). PA = phosphatidic acid; CL = cardiolipin; lipo = liposomes.
binding of this glycolipid with Aβ. The affinity of liposomal PA or CL for Aβ1-42 fibrils was in the nanomolar range, with values of 22–87 nM and 45–75 nM, respectively. Previously, the existence of an interaction, between Aβ1-42 and PA (either alone or embedded in liposomes) has been inferred from its effect on peptide aggregation [21]; however, the affinity was not evaluated and the investigation was carried out at concentrations much higher than those herein utilized. Much lower, or even negligible, binding of liposomes incorporating PA or CL was detected on immobilized Aβ1-42 monomers. Very interestingly, a significant binding was found onto a chip surface prepared with an oligomeric preparation of Aβ1-42. Since the oligomeric preparations contain a relevant proportion of monomers [31,32] which do not bind PA/CL liposomes, we can infer a very high binding to oligomers, possibly higher than the binding to fibrils. This is noteworthy, since oligomers are now identified as the most toxic Aβ1-42 species [7,8], likely appearing before plaque deposition in an early stage of AD pathology. The finding that PA/CL NPs did not show any binding to BSA has also relevant implications for the use of these NPs in vivo. In fact, the presence of plasmatic BSA is not expected to affect the interaction of PA/CL NPs with Aβ1-42 aggregates.

One striking finding coming from SPR results was that the binding of PA/CL liposomes to Aβ1-42 aggregates is pseudo-irreversible, with a very low dissociation rate constant (Koff). A complex model likely underlines this interaction, as clearly indicated by the impossibility to globally fit sensorgrams obtained with different liposomal PA concentrations. We suggest that the very high binding affinity observed with PA/CL NPs can be ascribed to multivalent interaction, i.e. different PA/CL molecules on the same NPs contribute to the binding to the immobilized Aβ1-42. It has been previously shown that a multivalent ligand (dendrimer [17]; nanoparticle [18]) has a binding affinity for its target which can...
greatly exceed the binding affinity of the same ligand, if monovalent. This “avidity” effect is well known for antibodies, and SPR studies showed that the affinity of specific IgG antibodies for immobilized Ab1-42 fibrils was 2–3 orders of magnitude higher than the affinity of the corresponding Fab [54]. This increase of affinity was due, in particular, to a decrease of the dissociation rate constants, approaching those of a pseudo-irreversible binding.

The fact the PA or CL confer the liposome a high affinity for Ab1-42, in all its aggregation forms, was also confirmed by our studies based on ultracentrifugation on a discontinuous density gradient, in which the interaction between the liposomes and the peptide occurred in solution. Also in this case, PA or CL liposomes show highest binding for fibrillar preparations of peptide, confirming the multivalent nature of the interaction. Our results suggest that NPs (liposomes or SLN) containing CL or PA might be used to target Ab1-42 with high affinity, and it could be interesting to check how the size of these NPs may affect their binding performances. Moreover, our data open the possibility to develop more sophisticated nanodevices possessing other important features. For instance, it will be very important to functionalize NPs with imaging probes and with molecules enhancing the passage through the BBB, and to load them with specific drugs [42,55]: the ability to trap calcein is a proof that PA/CL liposomes own this latter feature. The high performance to target Ab of the NPs designed in the present investigation is a pre-requisite for all the above issues. Also the high negative zeta-potential displayed by our NPs is predictive of a good stability, as shown by the observation that the size is stable for long times, indicative of their ability to resist aggregation. The importance of our results is also strengthened by the observation that the interaction occurs in physiological buffer and it is potentially not affected by the presence of BSA; this preliminary information is encouraging for a possible use in vivo that, of course, will deserve separate investigations. This strategy could be also extended to determine the ability of our NPs to bind Ab1-40 or other amyloidogenic peptides. Another possible advantage of our biomaterial is that PA and CL on their surface should not compete with their cellular counterpart for extracellular Ab1-42, which accumulates in the diseased brain and circulates in blood. In fact, endogenous PA and CL are confined mainly at the cytoplasmic side of the membrane and within the inner mitochondrial membrane [56,57] respectively. Finally, PA and CL can be considered lead compounds useful as starting point for chemical modifications in order to improve potency, selectivity, or pharmacokinetic parameters [58].

5. Conclusions

We report in the present investigation the preparation and characterization of lipid-based nanoparticles (liposomes and SLN)
targeting with very high affinity aggregated forms of Ab40-42 (fibrils and oligomers), opening the possibility to develop more sophisticated nanodevices to be exploited for diagnostic or therapeutic purposes. Currently, there are no efficient diagnostic and therapeutic tools for AD and our results could be important to set up a further experimental investigation in order to functionalize liposomes with imaging probes and with ligands able to allow the BBB passage.

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References


