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Targeted drug delivery across the blood brain barrier in Alzheimer’s disease

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Abstract

The discovery of drugs for Alzheimer’s disease (AD) therapy that can also permeate the blood brain barrier (BBB) is very difficult owing to its specificity and restrictive nature. The BBB disruption or the administration of the drug directly into the brain is not an option due to toxic effects and low diffusion of the therapeutic molecule in the brain parenchyma. A promising approach for drug systemic delivery to the central nervous system is the use of nanosized carriers. The therapeutic potential of certain nanopharmaceuticals for AD has already been demonstrated in vivo after systemic delivery. They are based on conjugates of drug and monoclonal antibodies against BBB endogenous receptors; cationized or end terminal protected proteins/peptides; liposomes and polymeric nanoparticles coated with polysorbate 80, cationic macromolecules or antibodies against BBB receptors/amyloid beta-peptides. Optimization and further validation of these systems are needed.
INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disorder of the human brain causing dementia. The majority of the 35 million people who have dementia worldwide are thought to have AD (World Alzheimer's Report from Alzheimer's Disease International). The initial clinical symptoms of the disease are almost imperceptible and typically involve lapses of memory for recent facts and poor judgment [1]. The performance of complex work tasks and ability to acquire new information may be reduced. After a couple of years cognitive functions are affected and patients show spatial disorientation, apathy, general disinterest and difficulty in performing simple tasks such as preparing meals or managing bank accounts. Patients frequently lose emotional control, which may be accompanied by physical or verbal aggression. Symptoms of depression may prevail in the early stage of illness. With progression of the disease, patients develop motor problems showing difficulty for walking and manual activities like writing. Recent memory is severely affected. Over several years, the disease leads to a gradual deterioration of the life of the patients, who manifest a marked dementia with profound memory and cognition losses. Many patients become immobile and succumb to respiratory difficulties.

AD is neuropathologically characterized by neuritic plaques and neurofibrillary tangles in regions of the brain particularly related to memory and cognition [2]. The neuritic plaques are spherical extracellular lesions that contain amyloid β-peptide fibrils surrounded by dystrophic axons and dendrites, activated microglia and reactive astrocytes. The amyloid β-peptide (Aβ) has 40 or 42 amino acids and is a normal metabolic product of enzymatic processing of a transmembranar protein, the amyloid precursor protein. The sequence of 40 residues is normally the more abundantly produced by cells. Amyloid plaques deposit in the brain parenchyma and around cerebral vessel walls. Diffuse plaques are also observed in the same brain regions in much larger number. They consist of amorphous extracellular deposits of Aβ (material that lack of fibrils). Diffuse plaques appear to represent an earlier stage of neuritic plaques. Neurofibrillary tangles are helical paired filaments composed largely of abnormal microtubular tau protein. These fibers are found in the cytoplasm of neurons. There are no peripheral biochemical markers for AD and confirmation still requires post-mortem observation of the classical lesions.

The existing therapies for AD are based on the alleviation of its symptoms. Four of the five drugs approved for clinical use as treatment of cognitive symptoms, donepezil (Aricept®), rivastigmine (Exelon®), galantamine (Razadyne®) and tacrine (Cognex®), are inhibitors of the acetylcholinesterase (AChE), raising the levels of acetylcholine, a neurotransmitter that is deficient in the brains of patients with AD. Tacrine has limited use due its severe side effects. The other drug, memantine (Namenda®) is an uncompetitive antagonist of the N-methyl-D-aspartate receptor, regulating the activity of glutamate, which is released by cells damaged by the disease. Drug development in AD includes several strategies such as immunotherapy and inhibitors of Aβ aggregation, which result from the growing knowledge of Aβ
generation and the effects of Aβ oligomers (soluble aggregates) on the synaptic function [3, 4]. The development of AD drugs is a very active research area and many promising molecules reach the clinical trials but they end up failing [5]. One of the main reasons for that disappointing outcome is the poor blood brain barrier (BBB) permeation of the drugs. The BBB is anatomically defined as the cerebral microvascular endothelium, which is different from all other vascular beds as it has tight cell-cell junctions and few alternate transport pathways (e.g. decreased pinocytotic activity and significantly decreased number of intracellular fenestrae). The brain endothelial cells have extra degrading enzymes and additionally express high levels of active influx/efflux membrane transport proteins (e.g. P-glycoprotein, Multidrug Resistance Protein-1, Breast Cancer Resistance Protein) that limit the permeability of molecules. The BBB has other cells anatomically associated to the endothelium in particular pericytes and perivascular astrocytes that contribute to the regulation and maintenance of the BBB function and characteristics [6, 7]. Physiological and biochemical features will also affect the transport of molecules to the brain [8, 9]. Thus in brain drug delivery, factors such as the binding of the drug to albumin in the peripheral blood and the complex barrier layers of the central nervous system (the brain endothelium, the arachnoid epithelium and the choroid plexus epithelium) have to be considered. Another relevant question of molecule transport in AD is the BBB dysfunction [9], although it is now generally accepted that the barrier remains intact in early AD.

Several strategies are being exploited for the delivery of AD drugs through the BBB [9-12]. In this review, the BBB drug-targeting strategies for AD that have been shown to have a therapeutic effect in AD transgenic rodent models and/or to increase the concentration of the drug in the brain of animal models after parenteral administration are discussed. These approaches include reengineering the drug molecule by coupling monoclonal antibodies (mAb) against BBB receptors, peptide cationization and/or other chemical modifications, and the use of liposomes and polymer-based nanoparticles coated with mAb, polysorbate 80 or trimethylated chitosan.

TRANSPORT OF DRUGS THROUGH THE BBB

Due to the tight junction between adjacent endothelial cells, most molecules are forced to cross the BBB via the transcellular route (Figure 1) [7, 13, 14]. Some small lipophilic molecules (molecular weight < 500 daltons) such as barbiturates and ethanol diffuse freely from the blood to the central nervous system. Other small molecules and macromolecules such as peptides and proteins are generally excluded unless they are essential molecules, which are transported through interaction with specific transporters and/or receptors expressed at the luminal (blood) side of the endothelial cells. The transcellular traffic of small hydrophilic essential molecules such as glucose, amino acids or nucleosides is regulated by specific transport proteins expressed at the luminal and abluminal membranes (carrier-mediated transport and active efflux transport). Large hydrophilic essential molecules such as peptides and proteins are
transferred by endocytosis in vesicles, which can be either specific (receptor-mediated transcytosis) or less specific (adsorptive-mediated transcytosis) [7, 13, 14]. Certain endogenous large-molecules such as hormones, insulin, transferrin for iron, lipoproteins are transported to the brain across the BBB via receptor-mediated transcytosis (RMT), which is the transport through specialized ligand-specific receptor systems expressed at the luminal and basolateral side of the brain endothelial cells. Polycationic proteins such as protamine and cationized proteins (proteins with a basic isoelectric point) can bind to luminal plasma membrane of the brain endothelial cell and be transported through the BBB by adsorptive-mediated transcytosis (AMT), also known as the pinocytosis route [15-17]. The transport is triggered by the electrostatic interactions between the positively charged proteins and negatively charged regions of the membrane surface of brain endothelial cells.


BBB drug-targeting approaches include invasive procedures (osmotic opening of the BBB, shunts and biodegradable implants), pharmacological approaches (modifying, through medicinal chemistry, a molecule) and physiological strategies that take advantage of the RMT and AMT pathways [14, 17]. Invasive procedures can be highly traumatic and often have low therapeutic efficiency with substantial side effects. Pharmacological approaches often result in loss of the central nervous system activity. RMT is the most accepted process in physiological strategy because of its specificity and the approaches comprise the association/conjugation of drugs to specific ligands that are recognized by the BBB endogenous receptors (Table 1). AMT-based strategies involve cationic liposomes/ nanoparticles, cell-penetrating peptides and cationization of proteins with diamine/polyamine (Table 1). The principal constraint of AMT is its lack of selectivity, which potentially can cause side effects of drugs in non-targeted organs.
Table 1. Specific ligands and other molecules used in physiological approaches to target drugs/ nanoparticles to the brain parenchyma through the BBB.

<table>
<thead>
<tr>
<th>Process</th>
<th>Molecules</th>
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<tr>
<td>Receptor mediated transport</td>
<td>Transferrin</td>
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<td>Monoclonal antibodies against transferrin</td>
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<td>receptor and insulin receptor</td>
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<td></td>
<td>CRM197, a non toxic mutant of diphtheria toxin</td>
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<td>Low-density lipoprotein receptor (LRP) related proteins:</td>
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<td></td>
<td>Melanotransferrin</td>
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<td>Receptor associated protein</td>
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<td>LRP binding domain of the apolipoprotein B</td>
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<td>LRP binding Angiopep (19 amino acids peptide)</td>
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<td>Adsorptive mediated transport</td>
<td>Diamine and polyamines:</td>
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<tr>
<td></td>
<td>hexamethylenediamine (synthetic)</td>
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<tr>
<td></td>
<td>putrescine, spermidine and spermine (naturally occurring)</td>
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<td>Cell penetrating peptides:</td>
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<td></td>
<td>Penetratin (derived from Antennapedia protein)</td>
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<td>TAT protein (HIV-1 trans-activating transcriptor)</td>
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<td></td>
<td>FBP (fusion sequence-based peptide)</td>
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<td></td>
<td>Syn-B (derived from a natural mammalian antimicrobial peptide)</td>
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<td>Poly-arginine peptides</td>
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**RE-ENGINEERING Aβ PEPTIDE AND AMYLOID ANTIBODIES**

The idea that amyloid plaques could be imaging *in vivo* by using labeled Aβ peptide came from the observation that radioiodinated Aβ40 binds specifically and reversibly to neuritic and diffuse plaques as well as cerebrovascular amyloid in brain tissues obtained from AD patients [18]. Studies using rats as animal models showed that the radiolabeled peptide was not able to cross the brain capillary endothelial wall [19]. Although Aβ40 was found to cross the BBB in other studies, the systemic factors proteolytic degradation and systemic clearance seem to be determinant in controlling the level of plasma Aβ [20-23]. Aβ peptides that circulate in the blood are assumed to adsorb to the brain microvasculature, nonspecifically, and to be rapidly metabolized without significant transport into the brain [5, 19]. The efflux of Aβ from the brain depends by its turn on the aggregation state of the peptide. Soluble Aβ monomers, but not dimers, are eliminated from the brain [24]. The strategies to delivering Aβ as an imaging agent to the brain include its conjugation to mAb or fusion antibodies against BBB receptors and polyamine modification (Figure 2). Radioiodinated Aβ was conjugated to mAb against receptors of the BBB to enhance its transport through the BBB [19, 25-29]. The conjugation of 125I-labeled Aβ40 (125I-Aβ40) to a murine mAb to the rat transferrin receptor (OX26) was achieved by the streptavidin-biotin technology [19]. A spacer-biotin linker was used for 125I-Aβ40 monobiotinylation followed by binding
to streptavidin-OX26 mAb. The OX26 was conjugated to streptavidin by a thioether linkage. The spacer linker contributed to minimize the steric hindrance that could prevent the binding of $^{125}$I-Ab$_{40}$ to amyloid plaques. Combining monobiotinylated $^{125}$I-Ab$_{40}$ and streptavidin-OX26 mAb increased the delivery of the peptide to the brain and decreased the Ab peripheral metabolism in anesthetized rats after intravenous (IV) injection [19]. The antibodies did not compromise the binding affinity of $^{125}$I-Ab$_{40}$ to amyloid plaques in frozen sections of AD brain. The OX-26 is specific for rats and aged rats do not show accumulation of amyloid plaques in the brain, hence the conjugation technology was extended to other mAb to validate the system in other animal models. Monobiotinylated radiolabeled Ab$_{40}$ was conjugated to 83-14 mAb to the human insulin receptor (animal model rhesus monkey) [25] and to RI7-217 or 8D3 rat mAb to the mouse transferrin receptor (animal model mice) [26, 27]. The work with primates has demonstrated that the complex $^{125}$I-Ab$_{40}$/83-14 mAb also effluxes from the brain to the blood with a half-time of 16 h [25]. More recently, a fusion protein composed of a chimeric mAb against the mouse transferrin receptor (cTfRmAb) and avidin (AV) was tested as a carrier of the radiolabeled Ab in rodent models [28]. The brain uptake of the complex monobiotinylated $^{125}$I-Ab$_{40}$/cTfRmAb-AV is on the order of 2% injected dose (ID)/gram brain at 60 min after IV injection and is close to the brain uptake of small molecules proposed as amyloid imaging agents based on $^8$F-labeled benzofuran derivatives (3% ID/gram at 60 min after IV injection) [28, 30]. A similar system was prepared using the fusion protein of chimeric mAb to the human insulin receptor (HIRmAb) and avidin (AV) as a neuro-imaging agent for humans [29]. These fusion proteins are prepared by genetic engineering a gene, which encodes a fusion protein formed by the amino terminus of avidin linked to the carboxyl terminus of the heavy chain of the mAb [31].

The polyamine modification of radioiodinated Ab$_{40}$ was achieved by amidation of the peptide carboxylic groups using a natural diamine (putrescine) and a water-soluble carbodiimide crosslinker that activates carboxyl groups [32, 33]. This modification increased the Ab$_{40}$ BBB permeability in a transgenic mouse model of AD after IV injection by at least twofold comparing to the labeled peptide and enhanced its binding to amyloid plaques in AD brain sections [32]. Gadolinium labeling was combined to the diamine modification to prepare an Ab$_{40}$ probe able to be detected by magnetic resonance imaging (MRI) [33]. The peptide was synthesized with the chelating agent diethylenetriaminepentaacetic acid (DTPA) at the amino terminus, followed by chelation to gadolinium (Gd) and covalent binding of the diamine to COOH groups using a carbodiimide crosslinker. An Ab$_{40}$ derivative based on the first 30 amino acid residues with amine-modified carboxyl groups of glutamic and aspartic acid and containing Gd-DTPA-aminohexanoic acid covalently linked to the N-terminal was subsequently proposed to overcome some problems associated with Gd-DTPA diamine modified Ab$_{40}$ (inherent problems of peptide cross-linking and decreased solubility during carbodiimide-mediated modification) [34]. Although several cationic proteins are known to cross the BBB by
AMT, the transport mechanism of diamine/polyamine-modified peptides/proteins has not yet been identified and may involve other processes such as carrier-mediated transport rather than simply electrostatic interactions [17].

**Figure 2.** Design strategies for transporting Aβ imaging agents through the blood brain barrier: A) Radiolabeled Aβ is bound to a linker domain, a 14-atom linker attached to biotin, which is bound by an avidin analogue such as streptavidin via a thiol-ether linkage (S) to the transport domain, which is comprised of either the OX26 murine monoclonal antibody (mAb) to the rat transferrin receptor (TIR), the 83-14 mAb to the human insulin receptor (HIR) or the R17-217 or 8D3 mAb to the mouse TIR; B) Radiolabeled Aβ is biotinylated at the amino terminus and is bound to a fusion protein, which is formed by fusion of avidin to the carboxyl terminus of the heavy chain of monoclonal antibodies, which is comprised of either the chimeric mAb against the mouse TIR or chimeric HIRmAb; C) Aβ is synthesized with the chelating agent diethylenetriaminepentaacetic acid (DTPA) at the amino terminus, followed by chelation to gadolinium (Gd) and covalent binding of the natural diamine (putrescine) to its carboxylic groups using a carbodiimide crosslinker.

Aβ peptides are also proposed as therapeutic molecules in active immunization. Transgenic AD mice immunized with Aβ42 by systemic injection developed anti-Aβ amyloid antibodies, which decreased the amyloid plaque number and improved cognition [35-38]. The passive immunization led to similar conclusions [39-41]. The mechanism by which the antibodies act seems to involve their transport through the BBB and direct interaction with amyloid plaques. This is supported by the fact that the binding of anti-Aβ antibodies to Aβ deposits results in their disaggregation [42, 43]. The problem is that this seems to be achieved at the expenses of the BBB integrity and is associated to life-threatening side effects. The BBB transport of anti-Aβ antibodies was attributed to the adjuvants associated to the vaccine, specifically the complete Freund’s adjuvant, which contains heat-inactivated Mycobacterium. The solubilization of the mycobacterial cell results in a high content of mannan polysaccharide, which leads to the formation of anti-mannan antibody, which, in turn, induces a reversible BBB disruption [44, 45]. Active immunization of AD patients in clinical trials was suspended due to problems related to the cerebrovasculature such as stroke and encephalitis [46, 47]. Passive immunization also causes side effects, mainly BBB disruption and cerebral microhemorrhage, which were attributed to the increase of Aβ plasma concentration [48-51]. The mechanism of antibody transport to the brain during passive immunization is not entirely understood [42, 52]. It has been suggested that anti-Aβ antibodies cross the BBB slowly by extracellular pathways (residual leakiness of the BBB) [9, 52]. Peripheral Aβ peptide may also be responsible for
transporting the antibody across the BBB since the complex anti-Aβ antibody/Aβ was found to be more permeable to the barrier than the antibody alone [53]. In this case, the transport of the antibodies would be dependent on the Aβ plasma concentration, which is highly variable from patient to patient. There is also the hypothesis that anti-Aβ antibodies act by sequestering Aβ peptide in the circulation, altering the Aβ equilibrium between the brain and plasma (sink hypothesis) [40]. Such diverse hypotheses result from the different methodologies and antibodies used in the studies. Nevertheless, the disaggregation of the amyloid plaques in the brain is only possible if the anti-Aβ antibody is able to cross the BBB, which may or may not occur during immunization [39, 54]. A fusion antibody against amyloid deposits was developed aiming the combination of the following functionalities: crossing the BBB bidirectionally; binding to Aβ amyloid deposits and disaggregating them [55]. The antibody is composed of a single chain variable fragment (ScFv) antibody directed against the amino-terminal region of the Aβ peptide and a genetically engineered chimeric HIRmAb (Figure 3). The anti-Aβ ScFv antibody was fused to the carboxyl terminus of the CH3 region of the HIRmAb heavy chain by a two amino acid linker (Ser-Ser linker) [55]. In the central region, the fusion antibody contains the CH2-CH3 interface of the human IgG constant region, which is the binding site for the neonatal Fc receptor (FcRn) [55]. It was established that the fusion antibody was able to cross the BBB in Old World primates (rhesus monkey) and to bind and disaggregate Aβ fibrils in vitro (double-antibody ELISA experiments) and in vivo (intracerebral injection into the frontal cortex and hippocampus in transgenic mice since the HIRmAb is not recognized by the rodent insulin receptor) [55]. The fusion antibody was designed in such a way that, in addition to its influx from the blood to the brain, it will also efflux from the brain. This was achieved by the CH2-CH3 interface to enable the binding and transport via the BBB FcRn receptor. It was proposed that the transport of IgG molecules from the brain to the blood is mediated by a BBB FcRn receptor [56]. There is also some evidence that the same receptor could be involved in the clearance of anti-Aβ antibody/Aβ complexes [42]. However, a recent work showed that IgG transport from brain to plasma still occurs in FcRn knockout mice [57]. Although, the experimental results of the efflux of the fusion anti-Aβ antibody from the brain seem to point out for FcRn receptor-mediated transport, these data still need validation [55, 57].

The polyamine modification has also been applied to anti-Aβ antibodies to increase their permeability at the BBB [15, 58]. A rat mAb (AMY33) directed against a synthetic peptide corresponding to the first 28 amino acids of Aβ was cationized with hexamethylenediamine [15] and a domain specific antibody fragment F(ab)’2 of a mAb against fibrillar human Aβ42 was covalently linked to putrescine [58].
Due to the limited translational applications posed by the covalent binding of proteins to carriers that are able to cross the BBB, a method based on a peptide transporter that does not require covalent linkage has recently been proposed [59]. The peptide consists of 16 lysine residues and 20 residues (151-170) of the low-density lipoprotein receptor (LDLR)-binding domain of apolipoprotein E (ApoE). The lysine residues meet the criteria of binding strongly and non-covalently to proteins. The domain of the ApoE mediates the BBB transport of the protein through LDLR-mediated transcytosis. The transport mechanism was not confirmed but the study demonstrated that the peptide carrier is able to deliver an antibody against Aβ to the brain of mice with AD after IV injection [59].

LIPID- AND POLYMER-BASED NANOPARTICLES

Liposomes and polymeric nanoparticles are widely used in the preparation of drug delivery system formulations for parenteral administration. Some of the liposome or lipid-based formulations are already in clinical use [60, 61]. They normally do not cross the BBB and are rapidly removed from the bloodstream by cells lining the reticulo-endothelial system (RES) but their pharmacological properties can be relatively easily modified [62-64]. For example, coating the systems with polyethylene glycol (PEG) increases their half-life in the circulation due to steric stabilization and BBB permeability modification can be achieved by modifying the system surface with antibodies that undergo RMT [14, 62-64]. In the study of nanoparticles for AD therapy different surface modifications have been tested: conjugation of monoclonal antibodies directed to the transferrin receptor of the BBB (OX-26) and against Aβ_{42} peptide to pegylated liposomes; coating polymeric particles with polysorbate 80, trimethylated chitosan or putrescine-modified F(ab')2 fragment of Aβ antibody (IgG4.1) (Figure 4 and Table 2). Liposomes and nanoparticles are versatile systems that can be used to encapsulate either lipophilic, amphiphilic or water-soluble molecules. The loading of the system needs always to be optimized according to the physico-chemical properties of the drug.
The liposome surface in the liposome cholesterol (liposomes conjugated appropriate length for stable insertion into the liposomal bilayer) [L cells the two antibodies were conjugated using the surfaces ratios) [carboxy(polyethylene glycol)-2000] (DSPE-PEG2000) and biotinylated DSPE-PEG2000 (20:10:0.8:0.015–0.065 mole ratios) were prepared and linked to streptavidin followed by coupling to two different biotinylated antibodies at their surfaces: one directed to the transferrin receptor of the BBB (OX-26) and other against Aβ1-15 peptide [67]. Both antibodies were conjugated using the streptavidin-biotin technology but no details were provided on how the binding of the two different mAb was controlled. The liposomes were taken up by immortalized human brain capillary endothelial cells hCMEC/D3 but their BBB permeability needs to be further validated by in vivo tests [67, 68].

Liposomes functionalized with Aβ short sequences were proposed as vaccines and were tested in mice model of AD [69]. Each terminal end of the sequence 1-15 of Aβ (Aβ15) was coupled to one lysine-palmitic acid conjugate (with the appropriate length for stable insertion into the liposomal bilayer) (Figure 4) and the amino acid residues 1–16 of Aβ was conjugated with one lysine-PEG-DSPC at each peptide terminal side (PEGAβ16). They were then reconstituted in liposomes consisting of dimyristoyl phosphatidyl choline (DMPC), dimyristoyl phosphatidyl glycerol (DMPG), and cholesterol (9:1:7 molar ratios) [69]. The vaccines differed only on the distance between the antigen and the lipid anchor in the liposome; whereas Aβ15 is close to the surface, the Aβ16 has a spacer (PEG with 77 units) that separates it from the liposome surface. This led to different immune response and memory restoration on APPxPS-1 double-transgenic mice. The system with Aβ15 induced an immune response that restored the memory of the mice, whereas the spacer prevented

Figure 4. Surface modification of lipid- and polymer-based nanoparticles for crossing the blood-brain barrier (BBB) to treat Alzheimer’s disease. Polymer nanoparticles were coated with polysorbate 80, trimethylated chitosan or putrescine-modified F(ab’)_2 fragment of Aβ antibody (IgG4.1). Liposomes were prepared with peptide immunogen – two terminal palmitoylated lysine residues were covalently linked at each end of Aβ15 – and pegylated liposomes were conjugated to monoclonal antibodies directed to the transferrin receptor of the BBB (OX-26) and against Aβ42 peptide (see Table 2 for details).

Liposomes

The liposome based-system better studied for BBB delivery is an immunoliposome made of distearoylphosphatidylcholine (DSPC), cholesterol and a PEG-derivatized phosphatidylethanolamine (PE) to which the OX26 murine mAb was coupled [14, 63, 65, 66]. Both in vitro and in vivo studies demonstrated that OX26-targeted liposomes cross the BBB. Because of these promising findings, the system is also being studied for application in AD therapy [67]. Liposomes of DSPC, cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy(polyethylene glycol)-2000] (DSPE-PEG2000) and biotinylated DSPE-PEG2000 (20:10:0.8:0.015–0.065 mole ratios) were prepared and linked to streptavidin followed by coupling to two different biotinylated antibodies at their surfaces: one directed to the transferrin receptor of the BBB (OX-26) and other against Aβ1-15 peptide [67]. Both antibodies were conjugated using the streptavidin-biotin technology but no details were provided on how the binding of the two different mAb was controlled. The liposomes were taken up by immortalized human brain capillary endothelial cells hCMEC/D3 but their BBB permeability needs to be further validated by in vivo tests [67, 68].

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this effect [69]. The explanation appears to be associated to the different conformation of the Aβ sequences and generated immunoglobulins, which were predominately of the IgG class for Aβ15 liposome and of IgM for PEG-Aβ16 liposome. The efficacy of the Aβ15 liposomes was assumed to be in part due to the fact that IgG antibodies could cross the BBB whereas IgM not.

**Polymeric nanoparticles**

Polymers that have been used to prepare nanoparticles for crossing the BBB include polybutylcyanoacrylate (PBCA), chitosan, poly(D,L-lactide-co-glycolide) (PLGA), poly(D,L-lactide) (PLA). PBCA nanoparticles coated with the nonionic surfactant polysorbate 80 (also known as Tween 80 or polyoxyethylene-20 sorbitan monooleate) were the first system proposed for drug delivery to the brain and have been intensely studied since then [70]. The commercially available AD drugs rivastigmine and tacrine were encapsulate in PBCA nanoparticles (Table 2) [71-73]. These drugs cross freely the BBB but a delivery system may improve their bioavailability in the brain and reduce their toxic side effects by releasing them in a sustained controlled manner. PBCA nanoparticles are normally prepared by anionic emulsion polymerization method by adding the monomer n-butyl cyanoacrylate to acidic medium containing a colloidal stabilizer (dextran) and coated with polysorbate 80. This system increased the concentrations of the cholinesterase inhibitors in the brain of healthy adult Wistar rats after IV injection by approximately 4-fold when compared to the free drug [71, 72]. The nanoparticle/rivastigmine formulation was studied in scopolamine-induced amnesia in mice model and showed to generate a faster regain of memory loss when compared to rivastigmine solution [73].

Polysorbate 80-coated PBCA nanoparticles were also used to encapsulate radiolabeled 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol), a copper/zinc chelator, and curcumin, a polyphenolic compound [10, 74, 75]. Clioquinol is known to bind with high affinity to amyloid plaques and to inhibit the Aβ accumulation in AD transgenic mice and thus it is proposed as a promising in vivo amyloid-imaging agent. The molecule is able to cross the BBB but shows neurological side effects. The polysorbate 80-coated PBCA nanoparticles enhanced the brain uptake of the radiolabeled quinoline in both wild type and AD transgenic mice [76]. The application of curcumin, which is reported to possess anti-amyloid and anti-tau hyperphosphorylation properties, is restricted by its insolubility in water. The encapsulated curcumin showed a mean residence time of 17 h and a concentration increase in the brain in healthy mice after IV injection when compared to the free drug (injection of nanoparticles at 5 mg/kg resulted in a maximum curcumin concentration of 34.1 ng/g in the brain after 0.083 hours, whereas the dose of 10 mg/kg curcumin solution led to levels of 25.7 ng/g after 0.5 hours) [75].

Polysorbate 80-coated PBCA nanoparticles were in addition proven to be effective in transporting contrast agents for in vivo optical imaging in AD transgenic mice after IV injection: Texas red, a molecular imaging dye that binds senile plaques ex vivo, and Trypan blue, a plaque-binding red fluorescing diazo dye that does not cross the BBB [77].
Adsorbed Alexa-488–conjugated anti-Aβ antibody (6E10) to the nanoparticle surface stained amyloid plaque 15 min after peripheral injection and peaking at 2 hours. A gadolinium (Gd)-based MRI contrast agent also adsorbed onto PBCA NPs was detected in the brain of wild-type mice: 5.34% of the injected nanoparticle-loaded gadolinium per gram of tissue in the brain compared with only 0.009% injected dose of free gadolinium per gram of tissue 2.5 hours after administration [77]. This work took advantage of the advanced imaging techniques such as four-dimensional real-time two-photon and MRI to confirm the brain uptake of polysorbate 80-coated PBCA nanoparticles, which occurs with a time constant of approximately 18 min. Polystyrene core/PBCA shell nanoparticles coated with polysorbate-80 and containing thioflavins, which bind to amyloid fibrils, were detected in APP/PS1 transgenic mice upon intracerebroventricular injection of the nanoparticle formulation but not after IV infusion [78].

The mechanism of BBB transport of polysorbate 80-coated PBCA nanoparticles is still debatable. It has previously been proposed that they are transported via RMT triggered by the adsorption of serum protein apolipoprotein E (ApoE) in the plasma but some evidences seem to contradict this mechanism [62, 79]. They are the following: i) ApoE adsorption is not specific to polysorbate 80 coated surfaces; ii) poly-(methylmethacrylate) or polystyrene nanoparticles coated with this surfactant are not delivered to the brain; iii) no brain uptake occurs with drug-polysorbate 80 control solutions. Because of such facts, a nonspecific BBB permeabilization mechanism related to the opening of the tight junctions between the brain endothelial cells and a synergistic toxicity effect of polysorbate 80 and PBCA nanoparticles was suggested [80]. Polysorbate 80 is known to cause BBB permeabilization and PBCA nanoparticles (coated or uncoated) were found to be toxic to mice and to induce permeabilization of an in vitro BBB model. However, the generalized toxic effect on the BBB by polysorbate 80-coated PBCA nanoparticles was not yet confirmed and instead specific mechanisms involving recruitment of the cerebral capillaries, stimulation of endocytosis and modulation of the tight junctions permeability have been proposed [81].

Chitosan is a polysaccharide consisting of N-acetyl-D-glucosamine and D-glucosamine monomers obtained by alkaline N-deacetylation of chitin, a polymer commercially extracted from shrimp shells. Because of its abundant availability, non-toxicity, low-immunogenicity and mucoadhesivity, chitosan is extensively studied in drug delivery. Nanoparticles of chitosan can be prepared by different methods including ionotropic gelation using a cross linker and have been proposed for the delivery of Aβ peptide fragments and tacrine [82, 83]. The brain uptake efficiency of Aβ fragments/chitosan nanoparticles was 80.6% but their BBB permeation might be associated to changes in BBB integrity since Freund’s adjuvant was also administered [82].

Chitosan nanoparticles were coated with putrescine-modified F(ab’)2 fragment of Aβ antibody (IgG4.1) by electrostatic interactions to target cerebrovascular amyloid deposits and brain parenchymal plaques [84]. The accumulation of 125I-labeled nanoparticles in the brain of Wild type mice following IV administration was 8 to 11 times higher than the
control system (chitosan nanoparticles coated with bovine serum albumin), although their plasma clearance was 9 times higher [84]. The pharmacokinetic data excluded the release of the antibody fragment from the particle surface in the plasma. In addition, in vitro studies demonstrated the transcytosis of the antibody-coated nanoparticles through a BBB endothelial cell model, which is attributed to the polyamine modification of the anti-amyloid antibody fragment [58]. The in vivo studies carried out for tacrine-loaded chitosan nanoparticles provided data concerning the drug distribution only in peripheral organs; neither the brain uptake nor the pharmacodynamic effects were evaluated [83].

PLA and PLGA are FDA-approved, biocompatible and resorbable via natural pathways. Because of that, they are considered the most promising biodegradable polymers for the preparation of nanoparticles including those for drug transport to the central nervous system. Nanoparticles made of PLA or PLGA are mainly prepared using the emulsion/solvent evaporation technique or the precipitation solvent diffusion technique and stabilized with PEG coating to reduce uptake by the RES. The cholinesterase inhibitors donepezil and rivastigmine were incorporated in PLGA particles and their pharmacodynamic effects were evaluated using mice or rat models [73, 85]. Although in vivo studies showed improvement in learning and memory after nanoparticle parenteral administration, the BBB permeation of the systems should be considered with precaution. PLGA nanoparticles, in the absence of surface modifications, are not expected to cross the BBB [62]. Since, in that works, the nanoparticles were not modified, a possible explanation for the observed effects is that PLGA nanoparticles reach the brain by adjuvant-mediated transport; the study with donepezil reports the co-administration of mannitol (5%), a hyperosmolar agent that disrupts the BBB, and polysorbate 80 (0.1%) [85]. Another possibility is that the nanoparticles allow the sustained release of the drug in the plasma and the free drug reaches the brain.

PLGA nanoparticles coated with trimethylated chitosan were shown to cross the BBB [86]. PLGA nanoparticles were prepared with coenzyme Q₁₀(Co-Q₁₀) as a model drug or with 6-coumarin as a fluorescence probe and trimethylated chitosan was conjugated to the particle surface by carbodiimide-mediated modification [86]. Fluorescence microscopy measurements showed that after IV injection in CD-1 mice the trimethylated chitosan-coated nanoparticles containing 6-coumarin were accumulated in the cortex, paracoele, the third ventricle and choroid plexus epithelium, whereas uncoated particles were not detected. Experiments in AD transgenic mice attested that the system with Co-Q₁₀ improves spatial memory and reduces the number of senile plaques. The positively charged coating is expected to facilitate the active transport of the particles via AMT [16, 87]. However, positively charged particles can be opsonized with plasma proteins leading to their rapid clearance and to the activation of the complement system initiating an inflammatory response. This might be prevented in part by adding PEG molecules to the nanoparticle surface.

Other polymeric system proposed for AD therapy that was tested in vivo is based on N-isopropylacrylamide, vinylpyrrolidone and acrylic acid. The nanoparticles were synthesized by free radical mechanism and loaded with
curcumin (NanoCurc™) [88]. After 16 h from the last intraperitoneal injection of the nanoparticles at a dose of 25 mg/kg twice daily and for 4 weeks in athymic mice the average curcumin concentration in the brain was 0.32 µg/g. The brain level of curcumin injected alone was not determined but it is described to be approximately 3 to 30 times less than the brain level of curcumin after NanoCurc™ administration. The results also showed that caspase 3 and caspase 7 activities were significantly decreased in the cortical lysate of NanoCurc™-treated mice, which may ameliorate pathological neuronal cell death observed in AD.

Table 2. Nanopharmaceuticals consisting of AD drugs and nanoparticles that were validated in rodent models by demonstrating their effective therapeutic potential and/or brain uptake in AD transgenic/healthy rodent models.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Cargos</th>
<th>Main observations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polysorbate 80-coated PBCA nanoparticles</td>
<td>Tacrine Rivastigmine</td>
<td>4-fold increase of the concentration in the brain of healthy adult Wistar rats compared to the free drug Improvement of the pharmacodynamics effects</td>
<td>[71-73]</td>
</tr>
<tr>
<td>Radiolabeled-clioquinol</td>
<td></td>
<td>Enhancement of the brain uptake in both wild type and AD transgenic mice after IV injection</td>
<td>[76]</td>
</tr>
<tr>
<td>Curcumin</td>
<td></td>
<td>Increase of the polyphenol concentration in the brain when compared to the free drug</td>
<td>[75]</td>
</tr>
<tr>
<td>Texas red Trypan blue Alexa-488–conjugated anti-Aβ antibody Gd-based contrast agent</td>
<td></td>
<td>Brain uptake with a time constant of 18 min Staining of amyloid plaques, cerebral amyloid angiopathy, and neuronal/glial cell bodies in living AD transgenic mice; 5.34% of nanoparticle-loaded Gd per gram of tissue in the brain</td>
<td>[77]</td>
</tr>
<tr>
<td>Chitosan nanoparticles</td>
<td>Aβ fragments</td>
<td>Administered together with Freund’s adjuvant A brain uptake efficiency of 80% in Kunming mice</td>
<td>[82]</td>
</tr>
<tr>
<td></td>
<td>F(ab’)2 fragment of Aβ antibody modified with putrescine (as coating)</td>
<td>Brain accumulation in Wild type mice following IV administration</td>
<td>[84]</td>
</tr>
<tr>
<td>Trimethylated chitosan coated PLGA nanoparticles</td>
<td>6-coumarin Coenzyme Q10</td>
<td>Accumulation in the cortex, paracoele, the third ventricle and choroid plexus epithelium in CD-1 mice after IV injection (with coumarin) Improvement of the spatial memory and reduction of senile plaques (with Co-Q10)</td>
<td>[86]</td>
</tr>
<tr>
<td>Nanoparticles of N-isopropylacrylamide, vinlypyrrolidone and acrylic acid</td>
<td>Curcumin</td>
<td>~0.322 ng curcumin/mg of brain tissue after 16 h of intraperitoneally injected</td>
<td>[88]</td>
</tr>
</tbody>
</table>
CHEMICAL MODIFICATION OF AD THERAPEUTIC PEPTIDES

The mechanism of action of peptides proposed for AD has been recently reviewed [89, 90]. The studies on AD therapeutic peptides focus mainly on the inhibition of Aβ peptide aggregation and neurotoxicity [90-92]. There is only one peptide described so far that targets tau protein [93]. Therapeutic peptides may be transported through the BBB by non-saturable pathways in sufficiently doses to affect the central nervous system but they are prone to rapid degradation by proteolytic enzymes, which will dramatically decrease their concentration in the blood. There are few works related to therapeutic peptides that were designed or modified to have prolonged plasma half-life time and proven to cross the BBB (Table 3). These peptides were shown to reduce the amyloid deposition in rodent AD models or to have potential therapeutic effect in AD patients enrolling clinical trials [89].

The 11 amino acid beta-sheet breaker peptide (iAβ11), developed based on the internal hydrophobic domain of Aβ peptide (residues 17-21, LVFFA) and on proline [94], was modified by polyamine covalent binding to improve its BBB permeability. In addition the L-amino acids were substituted to D-enantiomeric residues to increase its resistance to proteolysis in the plasma [95]. The iAβ11 was the starting point for the design of a pentapeptide, LPFFD, with increased efficacy and better BBB permeability owing to its reduced molecular weight but it turned out to be rapidly degraded after few minutes in the circulation. Several chemical modifications were performed to improve its proteolysis resistance, stability, solubility and BBB permeability and they include: N-terminal acetylation, C-terminal amidation, N-methylation and conjugation to PEG [96-99]. The sequence acetylated at the N-terminus and amidated at the C-terminus has been demonstrated to reach the brain of transgenic mice after injected intravenously [96, 98]. The additional N-methylation between proline and phenylalanine did not change the brain uptake rate of the peptide but improved significantly the enzymatic stability in rat brain homogenate [97]. The sequence LPFFD binds to Aβ peptide inhibiting its aggregation by blocking the formation of β-sheet conformation species and even the conjugation of long PEG chains (molecular weight 2000 or 5000 Da) did not block this binding affinity [99]. This β-sheet breaker peptide is known to disrupt amyloid fibrils, inhibit Aβ in vitro cell neurotoxicity and reduce cerebral amyloid deposition in vivo. The substitution of one phenylalanine with tyrosine and the amidation of the C-terminal carboxylate resulted in a sequence with high affinity for Aβ amyloid fibrils that was able of preventing neurotoxicity triggered by Aβ42, decreasing tau aggregation, crossing the BBB and protecting the synapses against the excitatory action of fibrillar Aβ42 [100-102].

Other β-sheet breaker peptide, based as well on the hydrophobic central Aβ peptide domain (residues 17-21), the sequence N-methyl-LVFFL with D-enantiomeric residues has reached clinical trials [103, 104]. The modifications allowed for increased biostability and prolonged elevated plasma levels. Aβ residues 12–28 where valine in position 18 was replaced by proline to avoid intrinsic toxicity associated with its residual capacity to form fibrils was proposed for the inhibition of the interaction between Aβ peptide and apolipoprotein E4 (ApoE4), which is considered a pathological
chaperone of Aβ conformational changes [105]. The sequence, synthesized with d-amino acids and end-protected (amidation of the C-terminus and acetylation of the N-terminus), reached the brain parenchyma of transgenic AD mice. The findings demonstrated that this sequence was able to block the interaction between Aβ and ApoE (competitive inhibition assay) and inhibit Aβ deposition in vivo, although the mechanism of action and the benefits of the approach of blocking Aβ-ApoE interaction for AD treatment are not clear [89, 105].

A small dipeptide that was designed by the principles of peptide chemistry for oligomer-inhibition (iterative selection cycles) was found to possess high serum stability and to cross the BBB [106]. The dipeptide consists of D-tryptophan, which is the aromatic recognition motif responsible for interacting with the aromatic core of Aβ, and α-aminoisobutyric acid (Aib), which is considered a strong β-sheet breaker (Cα-methylation β-breakage strategy). This dipeptide prevents the oligomerization of Aβ peptide, inhibits its toxicity Aβ towards cultured cells and reduces amyloid deposits in the brain of AD transgenic mice improving their cognitive performance [106].

Different mirror-image phage display screening using D-enantiomeric Aβ_{42} as a target under conditions where monomers or small oligomers of Aβ_{42} are the dominating species resulted in the selection of a D-peptide (D3) that has been shown to reduce amyloid plaque load and to improve the cognitive performance of AD transgenic mice after being orally administered [107]. D3 was shown to cross the BBB significantly in an in vitro BBB cell culture model suggesting that the peptide may be delivered to the brain but in vivo studies need to be carried out to confirm this hypothesis [108].

An apolipoprotein A-I (apo A-I) mimetic peptide (D-4F) that inhibits, in the presence of pravastatin, atherosclerotic lesion formation and decreases brain arteriole inflammation, was tested in transgenic AD mice and shown to reduce amyloid deposition [109]. Although an inflammatory component may contribute to the AD pathogenesis, the role of inflammation in the disease is not clear. D-4F was shown to be effective in AD mice after oral administration, lowering insoluble Aβ levels and improving their cognitive function. In addition, there was a decrease in the number of activated microglia and astrocytes, which are responsible for producing cytokines, oxidized lipids, and other pro-inflammatory molecules [109]. The mechanism of action of D-4F in AD mice is still not known. The peptide was shown to modulate the inflammatory properties of circulating lipoproteins, particularly HDL. But it was not determined if the peptide enters the brain as well as it is not clear if the peptide alone would inhibit Aβ deposition (D-4F was tested with pravastatin).
Table 3. Peptides that were shown to have a therapeutic effect and/or to reach the brain in AD transgenic animal models.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Strategy to improve the stability in blood</th>
<th>Main observations</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDLPFPVPID (iAβ1 or iAβ11)</td>
<td>All D-amino acids + covalent binding of putrescine (to increase BBB permeability)</td>
<td>Inhibition of Aβ fibrillogenesis</td>
<td>[95]</td>
</tr>
<tr>
<td></td>
<td>Only 6% degradation in rat plasma at 15 min after IV injection</td>
<td>5-7 fold increase in BBB permeability compared to non-modified sequence (note: for in vivo studies, Phe at position 5 was substituted with Tyr to allow radioiodination)</td>
<td></td>
</tr>
<tr>
<td>LPFFFD (iAβ5)</td>
<td>N-terminal acetylation + C-terminal amidation (Ac-LPFFD-NH₂) + N-methylation of amide between Pro and Phe (Ac-LP-(NMe)-FFD-NH₂)</td>
<td>Reduction in amyloid deposition and reversion of memory impairment in transgenic AD models</td>
<td>[96-98]</td>
</tr>
<tr>
<td></td>
<td>Replacing one Phe with Tyr + C-terminal amidation (LPYFD-amide)</td>
<td>Strong binding affinity to Aβ fibrils</td>
<td>[100-102]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protection against the synaptotoxic effect of aggregated Aβ in rat</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>~0.3% of the intraperitoneally injected dose reaches the brain</td>
<td></td>
</tr>
<tr>
<td>N-methyl-LVFFL-NH₂ (PPL-1019)</td>
<td>N-terminal methylation + C-terminal amidation + all D-amino acids</td>
<td>Prolonged elevated plasma levels</td>
<td>[103, 104]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Completed Phase I and Phase II clinical trial</td>
<td></td>
</tr>
<tr>
<td>NH₂-D-Trp-Aib-OH</td>
<td>D-amino acid + α-aminoisobutyric acid (Aib, selected for its β-sheet breaker activity, but is non-metabolized and crosses the BBB)</td>
<td>Reduction in amyloid deposition and improvement of cognitive performance of AD model mice</td>
<td>[106]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Orally bioavailable (39%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4–8%, depending on the route of administration, crosses the BBB</td>
<td></td>
</tr>
<tr>
<td>Ac-VHHQKLPPFAEDVGSNK-NH₂ (iAβ12-28P)</td>
<td>N-terminal acetylation + C-terminal amidation + All D-amino acids</td>
<td>Inhibition of Aβ/apoE interaction</td>
<td>[105]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduction in Aβ deposition in transgenic AD mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Plasma half-life is 62 minutes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BBB permeable</td>
<td></td>
</tr>
<tr>
<td>RPRTRLHTHRNR</td>
<td>All D-amino acids</td>
<td>Inhibition of Aβ toxicity in vitro</td>
<td>[107, 108]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduction of amyloid plaque load and improvement of the cognitive performance of AD transgenic mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BBB permeability assessed only by in vitro studies</td>
<td></td>
</tr>
<tr>
<td>Ac-DWFKAHYDKVAKFKEAF-NH₂</td>
<td>N-terminal acetylation + C-terminal amidation + All D-amino acids</td>
<td>Reduction in amyloid deposition</td>
<td>[109]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Improvement of the cognitive function in AD transgenic mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remains intact in the circulation after oral administration</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No studies on the BBB permeation</td>
<td></td>
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</tbody>
</table>
FUTURE PERSPECTIVE

The application of nanomedicines to the treatment and prevention of AD remains experimental. This review summarizes the systems that were shown to cross the BBB or that were validated in in vivo studies by demonstrating their effective therapeutic potential (Figure 2, Table 1 and 2). However many other nanopharmaceuticals have already been proposed for the treatment of AD. The considerable progress in research in drug delivery systems will resolve safety and toxicity issues and will contribute to the understanding of the mechanism of transport through the BBB. Effective methods of drug delivery to the central nervous system are expected within the next 10 years [110]. Future studies will contribute to the optimization of drug delivery systems that have already been shown to be effective in in vivo models.

The delivery strategies based on antibodies against the BBB endogenous receptors for AD diagnosis and therapy seem to preserve the BBB integrity. They are delineated to deliver the molecules by transcytosis either specific (RMT) or nonspecific (AMT). Further studies are needed to validate the transport mechanisms. In the case of immunotherapy, the antibodies should be engineering in such a manner that they are transported across the BBB and that they allow the complex anti-Aβ antibody/Aβ peptide to be rapidly cleared from the brain and from peripheral tissues [5].

Many nanoparticles already proposed for drug delivery to the brain such as pegylated liposomes coated with glutathione or exosomes (small membranous vesicles, 50–130 nm in diameter, that derive from the invagination of endosomal compartments secreted by most cell types) still need validation for AD treatment and they will certainly be considered. Significant results have already been achieved in respect to the functionalization or coating of nanoparticles. For example, the accessibility and flexibility of the specific ligand improve if it is attached at the terminus of PEG chains rather than directly bound to the surface of the liposome or nanoparticle. This is mainly due to the shielding effect of PEG chains, which prevent the interaction between the bound ligand and its receptor. Such effect can be reduced using PEG with low molecular weight (2000 or lower) but the more suitable strategy seems to be to link the vector directly to the PEG chains.

Concerning the delivery of AD therapeutic peptides, the strategies are common to that used to improve the half-life time of the class of peptide/protein drugs and basically are terminal modification (N-acetylation; C-amidation), substitution of L-amino acids with D-amino acids, conjugation to PEG and co-administration of specific enzyme inhibitors. However, these modifications should be carried out based on the knowledge of the enzymatic susceptibility of the molecule. For AD treatment, the peptides have to reach the brain parenchyma, thus additional modification may be necessary. Following the general tendency of AD drug development, the number of AD therapeutic peptides has significantly increased during the last years but studies on their delivery to the brain are deficient and need to be included in the early peptide discovery stage.
The effort to discover new drugs for AD should necessarily be combined with a BBB drug-targeting program to producing a cure, or at least a nanopharmaceutical that slows the progression of the disease. Above all, it is essential to focus on the optimization and validation of the systems that have shown promising results in preclinical studies.

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