Nanotechnologies for Alzheimer’s disease: diagnosis, therapy, and safety issues

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Abstract

Alzheimer’s disease (AD) represents the most common form of dementia worldwide, affecting more than 35 million people. Advances in nanotechnology are beginning to exert a significant impact in neurology. These approaches, which are often based on the design and engineering of a plethora of nanoparticulate entities with high specificity for brain capillary endothelial cells, are currently being applied to early AD diagnosis and treatment. In addition, nanoparticles (NPs) with high affinity for the circulating amyloid-β (Aβ) forms may induce “sink effect” and improve the AD condition. There are also developments in relation to in vitro diagnostics for AD, including ultrasensitive NP-based bio-barcodes, immunoassays, as well as scanning tunneling microscopy procedures capable of detecting Aβ1−40 and Aβ1−42. However, there are concerns regarding the initiation of possible NP-mediated adverse events in AD, thus demanding the use of precisely assembled nanoconstructs from biocompatible materials. Key advances and safety issues are reviewed and discussed.

From the Clinical Editor: This excellent review summarizes the impact of nanotechnology on the diagnosis and treatment of Alzheimer’s disease, ranging from circulating amyloid “sinks” to NP-based bio-barcodes and many other recent advances, without neglecting potential pitfalls, side effects and safety issues. A must read for anyone interested in the evolving interface of clinical neurosciences and nanotechnology. © 2011 Elsevier Inc. All rights reserved.

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Alzheimer’s disease (AD) is a devastating neurodegenerative disorder and the most common form of dementia among people over the age of 65 years. This neuropathological condition is characterized by a progressive loss of cognitive function and present two established pathophysiological hallmarks in the brain. These include extracellular accumulations mainly composed of amyloid-β (Aβ) peptide (also referred to as senile plaques) and intracellular neurofibrillar tangles of hyperphosphorylated τ protein.¹ Today, millions of people are affected by this neuropathology, posing a heavy economic and social burden. It is predicted that in the next few decades, AD will exert a huge societal and economic impact if no efficient therapeutic and/or early-diagnosis approaches become available. Moreover, considering the increase in population aging and survival, the impact of AD on the health care systems will be even more pronounced. Therefore, strategies for early detection as well as treatment of AD are among the most challenging and timely areas in modern medicine.

The blood-brain barrier (BBB) is a formidable gatekeeper in the body toward exogenous substances that maintains the chemical composition of the neuronal “milieu” for proper functioning of neuronal circuits and synaptic transmission. This barrier is formed at the level of the endothelial cells of the cerebral capillaries and essentially composes the major interface between the blood and the brain. The most important factor limiting the development of new drugs and biologics for the central nervous system (CNS) is the BBB. Generally, pharmaceuticals, including most small molecules, do not cross the BBB.² During the past decade numerous attempts have...
focused on this pivotal problem by designing different strategies that aid drug passage across the BBB. Among these, nanotechnology-based strategies have gained tremendous importance as some of them are capable of overcoming the limitations inherent to BBB passage. These include various types of lipidic, polymeric, inorganic, and other types of nanoparticles (NPs) for controlled drug delivery and release pertinent to various CNS conditions.3,4

A crucial challenge that is receiving increasing attention is to develop nanotechnology-based approaches for early diagnosis of AD. Early diagnosis could provide opportunities to treat patients at risk of AD development, thereby preventing the onset of irreversible neuronal damages. With respect to AD treatment, some strategies have been directed toward encapsulation of several types of biologically active molecules into NPs for their (targeted) delivery to the brain, whereas others have focused on the use of nanoconstructs to combat the toxicity of amyloid clusters by promoting their clearance or by altering their aggregation kinetics both in the brain and in the blood. Indeed, peripheral treatment with molecules that have high affinity for Aβ can reduce the level of Aβ in the brain through the sink effect. This approach can further benefit with engineered NPs exhibiting high affinity for Aβ, where sequestered plasma Aβ will be routed to hepatic and splenic macrophages for destruction. This approach could potentially reduce or prevent brain amyloidosis. It should also be emphasized that NPs can be introduced into the body through different routes of administration. Notably, some efforts suggest that orally delivered NPs can improve bioavailability of certain drugs used in AD. Accordingly, this article discusses current state-of-the-art nanotechnology-based approaches for AD diagnosis and therapy with a particular focus on the most significant recent reports and developments in the field.

Imaging-based nanotechnologies for AD diagnosis

Early diagnosis in AD (i.e., before clinical symptoms manifest) is crucial in preventing irreversible neuronal damages leading to dementia. Because the examination of living human brain is limited and invasive, the development of strategies to detect AD in its earlier stages is therefore essential. Since it is commonly accepted by the scientific community that the formation of senile plaques precedes the neurofibrillar degeneration, the majority of efforts are directed either toward detection and identification of amyloid plaques by magnetic resonance imaging (MRI) using NPs doped with contrast agents or, alternatively, by NPs tagged with fluorescent probes.

Iron oxide NPs

Magnetic iron oxide NPs have gained much interest because of their large surface area, magnetic properties, and limited toxicity. They have already been approved by the U.S. Food and Drug Administration (FDA) as MRI contrast agents in liver imaging.5 The synthesis of monocristalline iron oxide NPs (MIONs) covalently tethered to the N terminus of Aβ1-40 peptide through amide coupling and their development for the concomitant targeting and imaging of senile plaques has been reported.6 These MRI agents, by means of longitudinal µMRI, were able to recognize with high-affinity Aβ plaques in the brains of amyloid precursor protein (APP) and APP/PS1 transgenic mice when co-injected with mannitol (used to transiently open the BBB) (Figure 1). Although this study is very encouraging and demonstrates the proof of concept, manipulation of the BBB remains questionable for human testing.
A novel method for the selective labeling of Aβ1-40 fibrils has been reported with nonfluorescent, fluorescent rhodamine-tagged, or Congo red-encapsulated magnetic γFe₂O₃ NPs, even under competitive conditions (e.g., in the presence of human serum albumin). Moreover, these studies described the ability of iron oxide NPs to readily remove fibrils from solubilized Aβ aqueous sample by the simple use of an external magnetic field. 

**PE154 (heterodimeric acetylcholinesterase inhibitor)**

Beyond the well-known beneficial effects of acetylcholinesterase (AChE) for the treatment of AD symptoms (see “Delivery of Bioactive Molecules to the Brain”), recent studies have conferred upon this enzyme additional, nonclassical functions, including interactions with the Aβ peptide. Moreover, its structurally related inhibitor PE154 was also shown to act as a fluorescent probe for Aβ plaques present in tissue brain samples from both AD-mimicking triple-transgenic mice and humans with AD. This molecule, however, could not penetrate the BBB. To overcome this limitation, Härtig et al developed a carboxylated poly(glycidyl methacrylate) as well as PE154-loaded core-shell polystyrene-block-poly(n-butyl cyanoacrylate) (PS-b-PnBCA) NPs and demonstrated their ability to target Aβ deposits in vivo (Figure 2). Although NPs were injected in the hippocampus, this study provided the proof of principle regarding the Aβ targeting by using a fluorescent AChE inhibitor after its release from NPs in vivo. The authors even considered the possibility of developing the so-called theranostic approach for AD (i.e., a strategy combining both therapeutic and diagnostic approaches within a single nanodevice).

**Thioflavin T**

Thioflavin T (ThT) is a molecule capable of recognizing β-sheet structures related to Aβ aggregates both in vitro and in vivo. A recent attempt described the encapsulation of ThT into PS-b-PnBCA NPs, its release into the brain after intracerebral
injection, and its interaction with Aβ species, thereby showing clear visualization of amyloid aggregates.\textsuperscript{12}

**Quantum dots-Aβ complex**

Fluorescent semiconductor nanocrystals (quantum dots, QDs) have evolved over the past decade as highly useful fluorescent probes for biological labeling and diagnostics. QD features include long-term photostability and physicochemical stability, nanoscale size, and size-dependent emission wavelength.\textsuperscript{13} Tokuraku et al designed poly(ethylene glycol) (PEG)-QD-crosslinked Aβ peptide as a tool to monitor and to quantitatively describe the formation of fibrils and oligomers in solution and in a cellular system. This approach allowed the study of the Aβ peptide aggregation kinetics but could also be used to follow the in vivo peptide aggregation.\textsuperscript{14} Regarding the latter task, the authors have considered the functionalization of these nanoassemblies with appropriate ligands such as transferrin for BBB-crossing purposes.\textsuperscript{15}

**Gold NPs**

Gold NPs (Au NPs) also represent an interesting tool for studying Aβ peptide aggregation kinetics. Choi et al described the synthesis of heterodimeric NPs consisting of a cobalt(II) magnetic core and a platinum shell directly fused onto Au NPs and stabilized by lipoic acid–PEG coating.\textsuperscript{16} The terminal carboxyl groups of the PEG chains enabled covalent binding with lysine residues of neutravidin at the surface of the NPs. The Co@Pt-Au nanoassemblies presented a high magnetization value [63 emu g\textsuperscript{-1} (Co) at 3 T], making them appropriate for T\textsubscript{2}-weighted spin echo MR measurements. The MRI measurements of Co@Pt-Au-neutravidin NPs samples mixed with an increasing amount of biotinylated A\textsubscript{β\textsubscript{1-40}} peptides showed contrast changes governed by the peptide concentration. The results clearly showed that these NPs can be used in MRI to monitor key structural stages of Aβ self-assembly. In particular, a significant change in MRI signals during Aβ self-aggregation that corresponds to the detection of Aβ protofibrillar species in the early reversible stages of aggregation was observed. This methodology might also be important for the screening of Aβ anti-aggregating or disaggregating molecules.\textsuperscript{16}

However, several important parameters should be considered before a viable application is foreseen. Indeed, the intrinsic in vitro-in vivo cytotoxicity of the employed materials used to prepare the NPs should be thoroughly evaluated before further investigations. The feasibility of these approaches will further depend on developments that do not depend on invasive procedures.

**Nanotechnologies for detection of AD biomarkers in biological fluids**

The development of future effective treatments for neuronal degeneration will depend on early-diagnosis methods based on the detection and quantification of soluble AD biomarkers. In practice, two approaches are available: (1) the measurement of total τ protein and Aβ concentrations in cerebrospinal fluid (CSF) or plasma,\textsuperscript{17,18} and (2) the detection of suspected pathogenic biomarkers, such as the hyperphosphorylated τ protein and the Aβ-derived diffusible ligands (ADDLs, which are soluble oligomers). The first strategy is hampered by the significant crossing of τ protein and Aβ markers in biological fluids of healthy and AD subjects, and lead to inconclusive results,\textsuperscript{19} whereas the second, though more reliable, is strongly limited by the extremely low concentrations of the biomarkers that cannot be identified with enough accuracy by conventional enzyme-linked immunosorbent assays.

Remarkable results leading toward the development of new approaches for biomarker detection have been proposed by Georganopoulou et al, who developed an ultrasensitive NPs-based bio-barcode capable of detecting AD soluble biomarkers in CSF. The key feature of the system relied on the isolation of antigens (Ag) by means of a “sandwich process” involving oligonucleotide (DNA barcode)-modified Au NPs and magnetic microparticles (MMPs), both functionalized with monoclonal or polyclonal antibodies (Ab) specific to the ADDLs. Practically, an excess of Au NPs and MMPs (when compared to the ADDLs concentration) were mixed in a CSF sample; the recognition of the Ag from both particles led to the formation of the sandwiches that were then purified by magnetic separation (Figure 3, A). The strands of a dehybridized double-stranded DNA were isolated and easily quantified by a scanometric method using DNA microarray (Figure 3, B). The efficient Ag sequestration in solution and the amplification process resulting from the large number of DNA strands released for each Ag recognition allowed the system to identify ADDLs at subfemtomolar concentrations, thus improving the enzyme-linked immunosorbent assay test sensitivity by 6 orders of magnitude.\textsuperscript{20,21}

Another interesting procedure for Aβ detection was proposed by Chikae et al, who developed an electrochemical sensing protocol based on saccharide-protein interactions. Alkyne-terminated self-assembled monolayers were formed at the surface of Au NPs electrodeposited on screen-printed carbon strips and subsequently reacted with azido-functionalized sialic acid by “click” chemistry.\textsuperscript{22} The densely packed sialic acid domains were able to capture the Aβ peptide as a result of specific interactions, and the method enabled the detection of nonlabeled Aβ down to submicromolar concentrations. Importantly, the detection threshold of this technique was significantly lowered as compared with other techniques and thus represents an interesting step toward the development of a novel biomarker screening methodology.\textsuperscript{23}

The development of ultrasensitive immunosensors based on surface plasmon resonance (SPR) for Aβ\textsubscript{1-40} Peptide detection has also been reported. The procedure exploited the use of Au NPs-antibody fragment (fAb) complexes able to recognize Aβ peptide via the enhancement of the SPR signal. fAb able to specifically recognize the β-amyloid was anchored at the surface of Au NPs, and the Aβ-containing sample was flowed onto the chip. This was followed by a suspension of Au NPs-fAb, leading to plasmon signal generation. The procedure presented a linear correlation with the peptide concentration in a range of 9 orders of magnitude and increased the detection limit from 10 ng mL\textsuperscript{-1} to 1 fg mL\textsuperscript{-1}, when compared to a system on a bare gold substrate.\textsuperscript{24}

A parallel approach has recently been proposed by Kang et al, who developed an ultrasensitive electrical detection method for
Aβ1-42 using scanning tunneling microscopy (STM). Experimentally, a monoclonal antibody (mAb) fragment with high affinity for Aβ1-42 was immobilized onto a gold surface. Then, the sample containing the target biomolecule was deposited onto the mAb-functionalized surface, leading to its capture. Subsequently, Au NPs-fAb complex (also immunoreactive against the target) was reacted and conducted to the formation of “sandwich-like” structures. The resulting chip was finally analyzed by STM. It was shown that the surface density of the Au NPs correlated with the number of Ab-Ag binding events and that a successful Aβ detection was achievable down to 10 fg mL⁻¹ (Figure 4).²⁵

Haes et al designed a localized SPR (LSPR) nanosensor to detect ADDLs using scanning tunneling microscopy (STM). Experimentally, a monoclonal antibody (mAb) fragment with high affinity for Aβ1-42 was immobilized onto a gold surface. Then, the sample containing the target biomolecule was deposited onto the mAb-functionalized surface, leading to its capture. Subsequently, Au NPs-fAb complex (also immunoreactive against the target) was reacted and conducted to the formation of “sandwich-like” structures. The resulting chip was finally analyzed by STM. It was shown that the surface density of the Au NPs correlated with the number of Ab-Ag binding events and that a successful Aβ detection was achievable down to 10 fg mL⁻¹ (Figure 4).²⁵

Haes et al designed a localized SPR (LSPR) nanosensor to detect ADDLs as a potential biomarker for AD.²⁶,²⁷ The signal transduction mechanism of the LSPR nanosensor was based on its sensitivity to local refractive index changes near the surfaces of substrate-confined, size- and shape-controlled noble metals (Au, Ag) NPs.²⁸ Sandwich architectures were prepared by synthesis and immobilization of surface-confined Ag nanotriangles onto a mica surface using nanosphere lithography. Then, a self-assembled monolayer consisting of a mixed monolayer of 1-octanethiol for passivation of the NPs toward nonspecific binding and 11-mercaptoundecanoic acid for covalent attachment of Ab to ADDLs, was assembled on top of these Ag nanotriangles. The first Ab to ADDL was covalently attached onto the surface of the NPs via amidation reaction. The resulting biosensors were incubated with samples...
containing ADDLs, washed, and incubated with a polyclonal Ab solution specific to ADDLs to enhance the shift response for ADDLs. The LSPR nanosensor allowed analysis of biological species in a surfactant-free environment and was demonstrated to be sensitive enough for the detection of ultralow concentrations of ADDLs in biological samples. Moreover, this technique allowed the distinction between two different ADDLs species varying from their binding constant with the Ab specific to ADDLs ($7.3 \times 10^{12} \text{ M}^{-1}$ and $9.5 \times 10^{8} \text{ M}^{-1}$, respectively).

To the best of our knowledge, only one article has been devoted to the detection of $\tau$ protein, another specific AD biomarker, through a nanotechnology-based approach. Neely et al designed Au NPs coated with mAb specific to $\tau$ protein employed in a two-photon Rayleigh scattering assay, which enabled the detection of $\tau$ protein at concentrations greater than about 1 pg mL$^{-1}$. This concentration was about 2 orders of magnitude lower than typical $\tau$ protein concentration values (i.e., 195 pg mL$^{-1}$) in CSF (Figure 5). Moreover, the two-photon Rayleigh scattering assay showed a strong sensitivity for $\tau$ protein and was able to discriminate other proteins such as bovine serum albumin.

### Nanotechnologies for AD treatment

#### Delivery of bioactive molecules to the brain

A healthy BBB is a major obstacle for the development of both small and large neurotherapeutic molecules (e.g., recombinant peptides, Ab fragments, antisense oligonucleotides, viral vectors). In addition, the BBB also negatively affects drug efficacy and tolerance, because large doses of drugs are needed to reach levels above the minimum effective concentration in the brain. Nanoparticulate systems offer an opportunity to overcome such problems and can be used as “Trojan systems” for transporting active molecules across the BBB (Figure 6), thus reducing toxicity and improving therapeutic efficacy (Figure 7).

#### AChE inhibitors and acetylcholine (ACh)

The deficiency in cholinergic neurotransmission is considered to play an important role in the learning and memory impairment of AD patients. So far, cholinergic neurotransmission enhancement remains the most effective therapeutic approach to treat AD. Accordingly, rivastigmine, a noncompetitive and reversible inhibitor of both AChE and butyrylcholinesterase, was approved in 2000 by the FDA for the treatment of AD. Experimentally, this drug has been shown at least to maintain—if not to improve—cognitive function, global function, and behavior in AD patients. However, its clinical efficacy remains limited mainly due to poor brain translocation, which requires frequent injections, and its adverse cholinergic effects on peripheral organs.

The use of nanocarriers to transport rivastigmine to the brain might represent a promising alternative to circumvent these limitations. To this end, Wilson et al have encapsulated rivastigmine in polysorbate 80-coated polymeric PnBCA NPs.
with the aim of increasing the brain delivery of this compound and to reduce the side effects observed when the free drug is injected.\textsuperscript{4,35} The authors obtained a 3.8-fold rivastigmine uptake increase within the brain compartment compared to free rivastigmine after intravenous injection into rats. The mechanism(s) of NP translocation to the brain is related to polysorbate 80-mediated affinity for apolipoproteins E and A-I and subsequent NP internalization through low-density lipoprotein receptors (LDL-\(\tau\)) of the BBB. The same group also described a similar approach to increase the brain uptake of tacrine, another AChE inhibitor, using \(Pn\)BCA nanocarriers. In this case, the use of NPs increased the tacrine brain concentration by a factor of 4 when compared with the free drug.\textsuperscript{36} Recently, Joshi et al further reported positive therapeutic outcome in amnesic mice\textsuperscript{37} with rivastigmine encapsulated in poly(lactide-co-glycolide) (PLGA) and \(Pn\)BCA NPs.\textsuperscript{38}

Figure 5. (A) Schematic representation of the synthesis of Au NPs conjugated to mAbs specific to \(\tau\) protein and subsequent sensing of \(\tau\) protein based on mAb-conjugated Au NPs. (B) Transmission electron micrograph of Au NPs conjugated to mAbs specific to \(\tau\) protein before addition of \(\tau\) protein. (C) Transmission electron micrograph of Au NPs conjugated to mAbs specific to \(\tau\) protein after addition of 20 ng mL\(^{-1}\) \(\tau\) protein. Adapted from Neely et al.\textsuperscript{29} with permission of ACS publications.
An interesting approach for the delivery of ACh to the brain for AD treatment was proposed by Yang et al., using single-wall carbon nanotubes. However, single-wall carbon nanotubes are nonbiodegradable materials, and not much is known regarding their acute and chronic toxicity.

**Estrogens and androgens**

There is ample preclinical evidence that gonadal steroids (estrogens and androgens) play an important role in CNS development and functions. Estrogen treatment can decrease the risk of AD. Experimentally, estradiol may promote the growth and survival of cholinergic neurons and reduce significantly cerebral amyloid deposition. Taking this into consideration, Mittal et al proposed estradiol encapsulated in PLGA NPs as an alternative approach. By tuning the copolymer molecular weight and composition (i.e., the ratio between lactide and glycolide units in the co-polymer), they successfully increased the bioavailability of the drug after oral administration up to 10 times compared with the free drug. Likewise, mifepristone (11β-[4-dimethylamino]phenyl-17β-hydroxy-17[propynyl]estr-4,9-dien-3-one, more commonly known as RU 486), an active antiprogesterone compound, has been shown to slow the progression of cognitive decline in AD patients most likely via a mechanism related to P-glycoprotein transporter-mediated efflux of Aβ. Following encapsulation of mifepristone within PLGA NPs, He et al., evaluated the increase in drug bioavailability after oral administration. It was shown that NPs can significantly enhance the bioavailability of hormone and anti-hormone molecules. However, it should be emphasized that the biological effect of these NPs against the progression or development of AD still requires detailed evaluation.

**Curcuminoids**

Curcuminoids (Figure 7), obtained from *Curcuma longa* (turmeric), the most commonly used natural yellow photoconstituents in the food industry, have been widely screened in the past decade for biological activities such as anti-inflammatory, antioxidant (see also “Antioxidant Species” below), neuroprotective, hepatoprotective, anticarcinogenic, antiviral activities, and many others. Numerous investigators have reported that curcumin (Figure 7) can significantly reduce Aβ aggregate-related toxicity on neurons. Unfortunately, this compound exhibits poor stability as it is easily hydrolyzed under both acidic and alkaline conditions. It can also be oxidized or photodegraded, leading to poor bioavailability and thus negligible brain uptake.

**Non-functionalized NPs**

Two parallel studies investigated the encapsulation of curcumin into polymeric PnBCA NPs, and it was demonstrated that the encapsulation procedure dramatically increased curcumin half-life and concentration in the brain when compared with free curcumin. However, the therapeutic efficacy of this approach in AD models remains to be evaluated.

**Targeted NPs**

Another approach has utilized NPs decorated with appropriate ligands for curcumin brain delivery. This strategy was based on the preparation of curcumin-loaded PnBCA NPs decorated with ApoE3 ligands to exploit LDL-r-mediated transcytosis across the BBB and through SH-SY5Y neuroblastoma cells (Figure 8). The inhibition of Aβ1-42-mediated toxicity by ApoE3-functionalized nanocarriers was evaluated and compared with free curcumin on SH-SY5Y cells. The results indicated a significant reduction (40% compared with free drug at 100 nM Aβ) of Aβ1-42-related toxicity on cells treated with the functionalized nanospheres along with a reduction of reactive oxygen species formation.

**Immunotherapeutics**

Immunotherapy against Aβ1-42 peptide for AD treatment was tested earlier but has encountered severe complications.
(meningoencephalitis) during clinical trials. However, Agyare et al described the preparation of chitosan-based nanocarriers functionalized with pF(ab′)2.1, an Ab fragment modified with putrescine and specific to Aβ, that were able to cross the BBB and to target the brain amyloid deposits. These NPs could also be loaded with contrast agents for diagnosis purposes or with drugs able to reduce the amyloid plaques-associated toxicity.

Chelating ligands

There are suggestions that aberrant copper homeostasis has implications in AD. Accordingly, Treiber et al have engineered hyperbranched polyethyleneimine constructs with encapsulated Cu(II) ions, which were not only internalized by cells but also increased cytosolic concentrations of Cu(II) (by releasing the metal cations) and induced weaker Aβ turnovers. Unfortunately, no in vivo experiments have been conducted, presumably because of polyethyleneimine toxicity.

Further evidence supports the hypothesis that oxidative stress, generated by various mechanisms, may be among the major risk factors that trigger and promote AD. Oxidation reactions, catalyzed by metals such as iron (Fe(II)), copper (Cu(II)), aluminum (Al(III)), and zinc (Zn(II)), could take place if there is an increased local concentration of transition metals. Moreover, recent studies have shown that biometals mediate the deposition of Aβ in the CNS. Further confirmation arises from a study demonstrating that aggregated Aβ from postmortem AD brains could be resolubilized by co-incubation in the presence of such ion chelators.

Thus, metal-chelating compounds, such as ethylenediaminetetraacetic acid salts, desferrioxamine, and clioquinol, have been used to significantly improve the clinical conditions in AD patients. However, their poor brain uptake and the toxic side effects toward other sites have limited their systematic development as promising therapeutic agents. Because NPs represent potential carriers to transport drugs across the BBB, others have also developed synthetic strategies based on the covalent anchoring of metal chelators to NPs for CNS delivery. For instance, Cui et al demonstrated covalent grafting of D-penicillamine (see Figure 7) to lipidic NPs via disulfide bond, allowing the solubilization of Aβ-copper aggregates in vitro. Similarly, Liu et al reported the synthesis of a NP-MAEHP (2-methyl-N-(2′-aminoethyl)-3-hydroxy-4-pyridinone; see Figure 7) conjugate for interaction with Aβ aggregates. This study demonstrated the ability of these nanoassemblies to protect human cortical neurons from Aβ aggregate-associated toxicity and further reduced Aβ aggregate formation (Figure 9).

α-, β-, and γ-secretase inhibitors

Aβ peptides originate from proteolysis of the APP by the sequential enzymatic actions of β-site APP-cleaving enzyme 1 (BACE-1, a β-secretase) and γ-secretase (i.e., a protein complex with presenilin 1 at its catalytic core). Instead, the nonamyloidogenic pathway involves successive APP cleavages by α-secretase (thus precluding Aβ formation) and γ-secretase, leading to the formation of nonamyloidogenic fragments. The
disturbance of these two pathways and the aggregative feature of Aβ could be the triggering factor in AD.1

Accordingly, α-, β-, and γ-secretases can be considered as promising therapeutic targets.70 However, because of the multiple biological functions related to α- and γ-secretases, β-secretase might be the most relevant and attractive target.71 Despite several inhibitors and promoters transition states that have been proposed so far, an important limitation is still their delivery to the CNS. Several studies have described encouraging outcomes, but they were focused on intracranial injections of the inhibitors.72 In this context, a recent study by Smith et al suggested that the encapsulation of epigallocatechin-3-gallate (EGCG; also called gallic acid; see Figure 7), a natural α-secretase promoter, into lipidic NPs could increase its oral bioavailability.73 However, no additional information was provided regarding brain delivery and therapeutic efficacy of the described system.

Antioxidant species

Another strategy regarding the treatment of AD is directed toward the delivery of antioxidant species to the brain, because of their ability to quench the reactivity of reactive oxygen species (see also “Antioxidant Sponges” below).

Glutathione

The γ-glutamylcysteinylglycine (also called glutathione, GSH), a water-soluble endogenous antioxidant composed of glutamic acid, glycine, and cysteine (see Figure 7), is one of the most important intracellular antioxidants. It can protect cells from oxidative stress by scavenging singlet oxygen (¹O₂), hydroxyl radicals (HO·), and superoxide radicals (O₂·).74 Williams et al proposed the synthesis of PEG-GSH conjugates that self-assembled into NPs with the aim of increasing GSH levels in the brain. The authors described the ability of these nanoassemblies to alleviate the oxidative stress in SH-SY5Y cells against hydrogen peroxide (H₂O₂).75 A similar approach was proposed by Reddy et al, who investigated the encapsulation of a metalloprotein, superoxide dismutase (SOD), into PLGA NPs so as to increase its circulating half-life, cell membrane permeability, and brain uptake. SOD is a free-radical scavenger that plays a key role in the major endogenous cellular defense mechanism against superoxide radicals. The authors described the efficacy of these nanomaterials to deliver SOD to human neuronal cells in vitro and to protect them from H₂O₂-induced oxidative stress.76

Ferulic acid

Picone et al recently reported the use of solid lipid NPs (SLNs) as nanocarriers for ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, FA] due to their recently demonstrated ability to cross the BBB.77-80 FA, an antioxidant naturally present in plant cell walls, is obtained from the alkaline hydrolysis of curcumin and consists of a phenolic nucleus conjugated to an aliphatic unsaturated side chain (see Figure 7). This conjugated structure is favorable to radical scavenging because of its resonance-stabilized phenoxy radical, leading to strong antioxidant and anti-inflammatory activities.79 The authors demonstrated that FA-loaded SLN drug delivery devices were able to enhance the inhibition of neuronal oxidative stress and thus to block the cascade reactions leading to cellular death after the treatment of LAN5 human neuroblastoma cells with Aβ142 oligomers.80 This improvement compared with free FA was claimed by the authors to be due to the ability of the SLN nanocarriers to increase drug stability.
within biological fluids and to target specific organelles within cells, such as mitochondria.

Antioxidant sponges

Fullerenes

Specific carbon-based nanostructures have shown some promising therapeutic effects in AD. For instance, radical scavenging entities, such as carboxyfullerenes (C₆₀) could trap multiple radicals and have been consequently exploited as “radical sponges.” In this view, Dugan et al investigated the ability of water-soluble C₆₀ carboxylic acid derivatives, containing three malonic acid groups per molecule, to reduce the apoptotic neuronal death induced by exposure to Aβ₁₋₄₂. In this way, fullerenes could be an interesting alternative to reduce damage caused by Aβ toxicity.

Another widespread hypothesis about AD stipulates that calcium channels may play an important role in mediating Aβ activity on neurons. More precisely, the neurodegeneration could be mediated by an increase of Ca²⁺ influx caused by Aβ aggregates that would be able to create membrane channels permeable to Ca²⁺. Thus, Huang et al took advantage of the antioxidative effect of fullerol-1 on the in vitro reduction of Aβ-related toxicity and proved the existence of a mechanism governing this activity. Fullerol-1 was found to be able to attenuate the increase of intracellular Ca²⁺ concentration promoted by Aβ aggregates, either by interacting with the membrane lipid components and thus changing the membrane permeability, or by altering the lipid peroxidation and the membrane composition.

Nanocerias

Nanocerias (i.e., mixed-valence-state cerium) were used to drastically reduce the reactive oxygen intermediates intracellular concentration in vitro and in vivo, so as to prevent the loss of vision due to light-induced degeneration of photoreceptor cells. These results indicated that nanoceria particles were active for the inhibition of reactive oxygen intermediates-mediated cell death that is involved, among other species, in AD pathogenesis.

Physical interaction with Aβ peptide

Protein and peptide conformation is often altered following adsorption onto the surface of NPs, and this may affect their biological functions accordingly. This has been utilized to advantage by designing brain-specific NPs with affinity for Aβ peptides, thus affecting their aggregation or nucleation.

Considering recent findings about the toxicity of Aβ aggregates, an exogenous material that would be able to reduce the peptide toxicity may act following two opposite, postulated mechanisms: (1) by decreasing monomer nucleation and hence blocking the aggregation, which would result in a reduction of the formation of oligomers, fibrils, and plaques; or (2) by disaggregating amyloid plaques or fibrils (although they are currently not considered as the Aβ neurotoxic species) (Figure 10).

Polymer NPs

Cabaleiro-Lago et al reported the use of 40-nm-diameter poly[(N-isopropylacrylamide)-co-poly(N-tert-butylacrylamide)] (PNIPAAm-co-PtBAM) NPs to hinder Aβ fibril formation. The authors demonstrated that these NPs were able to interfere with the aggregation process by delaying, or even blocking, the nucleation step, whereas no influence on the elongation step was noticed. More importantly, it was found that the oligomerization of the peptide could be reversed sufficiently where mature fibrils start forming. These co-polymeric NPs introduced a “lag phase” in between the nucleation and the elongation steps of the fibrillation. This “lag phase” was shown to be strongly dependent on the physicochemical characteristics of the NP surface and concentration. These results might help to better elucidate the aggregation process of the peptide and to design NPs with optimized surface properties directed toward AD treatment.

The ability of sulfonated, sulfated, and fluorinated PS NPs in suppressing Aβ oligomerization and its toxicity toward cultured...
neurons has also been demonstrated. In addition, it was shown that hydrogenated NP counterparts had less efficacy, leading to \( \beta \)-sheet structures formation and aggregation. These results highlighted the crucial importance of the surface features of the NPs employed. Interestingly, Linse’s group recently demonstrated a dual effect of cationic amino-functionalized PS NPs toward the A\( \beta \) fibril aggregation process. The possibility to alter the peptide aggregation simply by tuning the NP concentration was well described, hence highlighting the pivotal role of NP dosing on the aggregation behavior. PS NPs offer an interesting model for optimization of surface properties for suppressing A\( \beta \) oligomerization. However, they are nonbiodegradable and may induce adverse reactions, but these strategies may be translated to biodegradable entities.

The very first example of biodegradable NPs able to bind the A\( \beta \) peptide and to inhibit its aggregation kinetics was reported in 2010 by Brambilla et al. The authors used PEGylated poly(alkyl cyanoacrylate) NPs based on poly[methoxypoly(ethylene glycol)-co-(hexadecyl cyanoacrylate)] [P(MePEGCA-co-HDCA)] co-polymer and monitored interaction with the A\( \beta \)\(_{1-42} \) peptide by capillary electrophoresis coupled to laser-induced fluorescence (CE-LIF) (Figure 11).

The principal advantage of this technique, when compared with other more routinely employed approaches such as ThT spectroscopy and SPR, is that NP interaction with peptide solution can be followed at nanomolar-range peptide concentrations. Through this approach, not only was the affinity constant measured, but the methodology further highlighted the crucial role of PEG chains at the surface of the NPs for peptide interaction. Experiments are continuing to clarify the exact role of PEG chains as well as to develop functionalized polymeric NPs by means of click chemistry, with higher affinity for the peptide. These NPs are potent candidates for suppressing A\( \beta \) aggregation as they were previously shown to cross the BBB.

**Liposomes**

Gobbi et al reported on preparation of liposomes and SLNs incorporating either phosphatidic acid (PA) or cardiolipins as a way to target the A\( \beta \) peptide. SPR investigations demonstrated that both PA-cardiolipins containing liposomes and SLNs displayed a fairly high affinity (e.g., 22–60 nM) toward chip-immobilized A\( \beta \) fibrils, probably due to a multivalent interactions effect. Very recent studies from Mourtas et al also reported the functionalization of azido-decorated liposomes with an alkyne-derivatized curcumin (see “Curcuminoids” above) ligand by 1,3-dipolar Huisgen cycloaddition reaction (the so-called click chemistry). The authors found out by SPR experiments that the liposomes decorated with the planar curcumin had the highest affinity constant (in the 1–5 nM range) reported thus far for A\( \beta \) fibrils, whereas nonplanar curcumin-decorated liposomes did not show any binding. As for the polymer NPs, these systems could pave the way for the development of colloidal systems able to capture A\( \beta \) and to reduce its inherent toxicity.

**Inorganic NPs**

Another interesting approach was proposed by Kogan et al, who demonstrated, by means of various complementary techniques, the possibility to locally and remotely heat and dissolve the A\( \beta \)\(_{1-42} \) deposits via the combined use of weak microwave fields (100 mW, 6 times weaker than those used by
conventional mobile phones) and Au NPs without any bulk effect. Although this approach was promising, it led to the formation of monomers and soluble oligomers, which are now considered as the most neurotoxic species in AD.\(^\text{102,103}\)

PEGylated micelles

Pai et al reported the ability of PEGylated phospholipidic micelles to interact with A\(\beta_{1-42}\) and to inhibit its aggregation (Figure 12).\(^\text{104}\) The authors proposed a double mechanism to explain PEGylated micelles activity. In the extracellular medium, these micelles would first interact with the peptide so as to bury its hydrophobic domains in the hydrophobic core of the micelle via a favored \(\alpha\)-helical conformation that prevents its self-aggregation. Second, the PEGylated micelles would shield the exposed hydrophobic domains of small A\(\beta_{1-42}\) aggregates with their hydrophobic acyl chains, thus avoiding further formation of aggregate-aggregate or aggregate-monomeric A\(\beta_{1-42}\).

Nanogels

Nanogels represent a promising class of drug delivery devices because of their high loading capacity, their high stability, as well as their responsiveness to environmental factors, such as ionic strength, pH, and temperature.\(^\text{105}\)

In this view, Ikeda et al suggested an original use of cholesterol-bearing pullulan (CHP) nanogels with a diameter of 20–30 nm as artificial chaperone systems for controlling the aggregation and cytotoxicity of A\(\beta_{1-42}\). These colloidal nanomaterials were able to efficiently incorporate the monomeric peptide and to inhibit its aggregation, thus suppressing its related toxicity against PC12 cells.\(^\text{106}\) Recently, the same group evaluated the ability of these nanogels to interact with the A\(\beta_{1-42}\) oligomeric forms and to reduce their toxicity on primary cortical and microglial cells. In vitro experiments indicated that CHPs prevented A\(\beta_{1-42}\) oligomers toxicity (Figure 13) and did not accumulate into lysosomes within the first 30 minutes.\(^\text{107}\) Further experiments on transgenic animals mimicking conditions of the AD neurological disorder are continuing, even if the ability of these nanostructures to surpass the BBB is still unproved. The concept developed with these CHP nanogels is very interesting if one considers the internalization of more specific A\(\beta\)-targeted ligands within the gel network.

Dendrimers

Dendrimers, as distinguished from hyperbranched polymers, are commonly considered as nearly monodispersed macromolecules constituted by a regular and highly branched three-dimensional architecture displaying a well-defined number of spatially arranged peripheral functional groups. They are generally produced through an iterative sequence of reaction steps, in which each additional iteration affords higher generation materials. Regarding these properties, dendrimers have gained an increasing interest in pharmaceutical science as drug carriers and as contrast agents.\(^\text{108}\) In particular, the possibility of functionalizing the peripheral groups with ligands of interest is an attractive strategy to study the physical interactions of these macromolecules with the A\(\beta\) peptide.

Several studies on the aggregation process of A\(\beta\) identified the critical peptidic sequence involved in amyloid aggregates formation. The hydrophobic core from residues 16–20 of A\(\beta\), the so-called KLVFF sequence, is crucial for the formation of \(\beta\)-sheet structures.\(^\text{109,110}\) It was also demonstrated that this peptidic region binds to its homologous sequence in A\(\beta\) and prevents its aggregation into amyloid fibrils.\(^\text{111,112}\) This sequence has been employed as a key compound for the development of inhibiting agents for preventing A\(\beta\) aggregation in vivo.\(^\text{109,113}\) By exploiting the above-mentioned properties of this peptidic sequence, Chafekar et al reported the synthesis of KLFFF-functionalized dendrimeric scaffolds and their marked inhibitory effect on A\(\beta_{1-42}\) aggregation, as well as their ability to disassemble preexisting amyloid aggregates. The same authors clearly demonstrated that these nanodevices exploited the multivalency feature of dendrimers to drastically enhance the affinity and specificity of KLFFF sequence toward A\(\beta\).\(^\text{114}\)

Several independent studies also suggested that A\(\beta\) was able to bind cells via an interaction with glycolipids or glycoproteins present at the external surface of the cellular membrane. It was also shown that the interaction affinity increased when gangliosides or sialic acid molecules were clustered on the cell surface.\(^\text{115-121}\) Based on this evidence, Patel et al synthesized sialic acid-conjugated polyamidoamine (PAMAM) dendrimers as membrane clusters mimetics to create A\(\beta\)-binding competing agents. The authors investigated the affinity constant between sialic acid-functionalized PAMAM dendrimers and A\(\beta\). They demonstrated the ability of these dendrimers to significantly reduce the A\(\beta\)-induced toxicity compared to nontreated control cells and cells treated with free sialic acid.\(^\text{122}\) Further experiments from the same group also reported that the positioning of the covalent bond between the dendrimer and the sialic acid was crucial regarding the modulation of the biological activity of the resulting conjugates. The addition of a spacer between the anomeric hydroxyl position of sialic acid and the dendrimer shell end groups resulted in an attenuation of the A\(\beta\) toxicity at lower concentrations compared to other binding strategies, thus highlighting a better match with the physiological attachment of sialic acid to cell membranes.\(^\text{123}\)
Proteinaceous fibrils are normally associated to other cell substructures or biological compounds, such as cell membranes or glycosaminoglycans, respectively. Glycosaminoglycans entities, such as heparin, which consists of linear arrangements of polysaccharides, have been shown to be particularly important in promoting the Aβ aggregation process. Thus, Klajnert et al discovered that the heparin-induced aggregation of Aβ1-28 could be modulated by the presence of generation 3 PAMAM dendrimers. In particular, the authors demonstrated that low concentrations of dendrimers reduced peptide aggregation, whereas higher concentrations had the opposite effect. Further experiments from the same group showed that the ionization state of acidic and basic residues of Aβ1-28 fragment played an important role regarding the interactions between the dendrimer shell and the Aβ1-28 amino acids, which can result in an enhancement or a decrease of the peptide amyloidogenicity. Despite experimental conditions far from that of the physiological environment, these studies showed that dendrimers can be exploited as a powerful tool for investigating the formation mechanism of amyloid-like structures.

Nanotechnologies for in vitro evaluation of drug activity

Although in vivo pharmacological assays remain the best way to evaluate drug activity and toxicity before clinical studies, they are time-consuming and expensive. Thus, a great deal of effort has been focused to establish efficient procedures for in vitro drug screening before in vivo preclinical experiments. Accordingly, a new drug sensitivity method based on the electrochemical behavior of an AChE biosensor has been reported by Du et al, based on Au NPs encapsulating a sol-gel silicate matrix. This system provides a stable and biocompatible environment for AChE biosensor immobilization onto an electrode surface and appropriate conductivity properties to the network that favor interfacial enzymatic hydrolysis reaction. Two different AChE
inhibitors (i.e., neostigmine and galantamine) were used to verify the proof of principle of this methodology. Here, electrosensitive substances formed after reaction of substrate with the encapsulated AChE amplify the biosensor sensitivity.

A method for ACh analysis based on its electrocatalytic oxidation on carbon paste electrodes modified with copper NPs has also been proposed. Experimentally, the electro-oxidation of ACh is mediated by Cu(II) active species, and the oxidized forms are detected and quantified using amperometric procedures. This technique was successfully applied for the quantification of ACh with high sensitivities in the micromolar range.128

Interestingly, the method developed by Haes et al.,26-27 based on LSPR (see “Nanotechnologies for Detection of AD Biomarkers in Biological Fluids” above), could also represent a very useful tool for the study of interactions between pharmacuetics and ADDLs.

Safety issues

Advances in nanotechnology and its applications in medicine have promoted serious issues in relation to NP-mediated toxicity and adverse reactions.129,130 This is of particular concern for intravenously injected AD nanomedicines, whether they are used for the induction of the sink effect or for reaching the brain for diagnostic imaging or therapeutic purposes. Notably, NP size, shape, and surface characteristics can modulate pharmacokinetics and biodistribution.129,131 Investigation in this area of research is still scant and particularly in relation to the brain. However, from the therapeutic point of view, attention must be focused on the benefit-to-risk ratio, and this is further dependent on NP dose and on the frequency of dosing. From the cytotoxicity angle, some constituents of polymeric NPs and nanoconstructs may inhibit the function of P-glycoprotein efflux pumps expressed at the luminal side of the brain capillary endothelial cells.132,133 This could potentially modulate or interfere with transport of hemostatic mediators in the CNs.134 Moreover, internalized NPs, depending on their nature and intracellular trafficking, could induce necrotic or apoptotic cell death through different pathways—a feature that is most prominent with polycationic constructs.129-135 Polymers also seem to be able to modulate gene expression,136 and this could pose serious problems in terms of nucleic acid delivery to the brain capillary endothelial cells with polyplexes and polycationic nanoconstructs. Indeed, this phenomenon has been associated with low expression of adenosine triphosphate-binding cassette genes after polymer treatment in some cells.139 In cytoplasm, polymers and partially degraded NP constituents may bind to endogenous nucleic acids such as double-stranded RNA and microRNA, resulting in interference with normal cellular regulatory processes and triggering off-target effects. This possibility may account for the reported polycation-specific induced “gene-signature.” A recent report has also raised concern over potential NP-mediated DNA and chromosome damage to tissues located behind cellular barriers through complex intercellular signaling processes.140 Therefore, some NPs may induce cell damage across an intact biological barrier without having to cross the barrier.

Recent evidence with clinically approved intravenous nanomedicines attests to initiation of hypersensitivity reactions in certain individuals, and these have often been correlated with activation of the complement system, which is the most ancient component of innate human immunity.141 The reactions are not mediated by immunoglobulin E and are usually associated with flushing and cardiovascular disturbances. Neurpsychosomatic and vegetative responses have also been noted in animals following polymeric and lipid-based nanomedicines.141 A wide range of polymers and nanoentities such as certain classes of liposomes, polymeric nanospheres, carbon nanotubes, and metallic NPs, whether stealth or not, can trigger complement activation via one or more of the three established initiation pathways (classical, alternative, and lectin pathways) that all converge at the step where the central complement protein C3 is cleaved.141-145 These pathways use different recognition molecules to sense a foreign particle, but use similar activation mechanisms to generate enzymes that cleave C3 (known as C3 convertases). The prime consequence of NP-mediated complement activation is surface opsonization by the opsonic fragments of C3 cleavage such as C3b and iC3b.131,144 This aids material recognition and rapid clearance by macrophages of the reticuloendothelial system bearing complement receptors (e.g., hepatic Kupffer cells, splenic marginal zone and red-pulp macrophages, blood monocytes) and therefore may be beneficial for intravenous nanomedicines that are intended to induce sink effect in AD.

Activation of the complement cascade further generates potent anaphylatoxins (C4a, C3a, and C5a), and these can trigger the release of secondary mediators from a wide range of immune cells that subsequently initiate anaphylaxis in sensitive individuals.131 Additionally, C5a is a potent chemotactic agent for monocytes, neutrophils, and a subset of T cells. It is also a powerful neutrophil activator increasing adhesiveness, stimulating the respiratory burst and degranulation. Such modulation of immune cell activities may further contribute to anaphylaxis and further complicate AD. Once C5 has been cleaved, the lytic membrane attack complex (C5b-9 or MAC) is assembled from the terminal complement components C5, C6, C7, C8, and C9. Remarkably, this complex also has the capacity to elicit nonlytic stimulatory responses from vascular endothelial cells and modulate endothelial regulation of hemostasis and inflammatory cell recruitment.131

The complement system is strongly activated in AD brain—particularly at the site of the senile plaque—and works in conjugation with activated microglia, which express high levels of complement receptors.146 Most attempts have detected MAC on dystrophic neurites and neurofibrillary tangles adjacent to the senile plaques, indicating autolytic attack and neurite loss.146 Complement proteins and activation products have also been found to be associated with cerebrovascular Aβ.147 However, only the earliest steps of complement activation appear in diffuse plaques containing nonfilamentous Aβ in many regions of the brain, including those generally not affected by the disease (e.g., cerebellum). Complement activation therefore exacerbates the pathology of AD. Recent evidence also attests that the classical pathway components (e.g., C1q) may be greatly upregulated in AD and particularly in the cortex.148 The binding interaction between Aβ and C1q is mainly ionic and occurs between the first 11 predominantly ionic residues of Aβ and residues 14–26 of the A chain of the collagen-like tail of C1q, which are mostly
cationic. Aβ interactions with C1q also lead to increased amyloid aggregation. Therefore, it remains essential that nanoengineering strategies that allow particle delivery into the brain do not induce further complement activation (notably activation of the terminal pathway) and particularly through C1q-dependent triggering mechanisms. Future efforts may further concentrate in design of brain-specific NPs that can also release complement inhibitors and particularly those that can block binding to the collagen tail of C1q, because this will not disturb Ab attack against an infectious agent. The complement system therefore plays a central role in nanomaterial and nanomedicine performance, and better understanding of material properties in relation to complement activation remains pivotal for precision engineering of highly effective nanomedicines for long-term management as well as treatment of AD.

Finally, with respect to the NP-mediated initiation of the sink effect, particular attention must be paid to the frequency and intervals between injections. Adverse responses may arise from excessive dose-dumping in macrophages (which may be selective and based on aggregate sizes) and initiation of immunogenic responses following repeated administration, resulting in altered NP pharmacokinetics and diminished efficacy.

Conclusions

During the past few years we have witnessed promising developments in relation to passive and active drug delivery to the brain using NPs. In parallel, remarkable nanotechnologies have emerged that can manipulate Aβ aggregation both in the brain and in the peripheral circulation, thus aiding experimental AD therapy.

However, three important open questions remain to be answered before engaging in further research toward clinical investigations: (1) the efficiency of symptom alleviation by these nanoparticulate systems must be validated in representative AD in vivo models, (2) FDA-approved macromolecules for nanoconstructs must be employed, and (3) noninvasive administrations of NPs must be considered for repeated and prolonged therapeutic purposes. These requirements are also relevant when considering the development of a strategy based on the production of NPs for physical interactions with Aβ peptide and/or τ protein. Moreover, in this case the majority of the above-discussed studies were performed in buffer environments, a considerable simplification of physiological conditions.

Recent achievements also described the design and the use of imaging agents and drugs based on nanoparticulate systems. However, the most prominent limitation is that plaques are no longer considered to be the toxic species in AD, although the majority of described methods are based on their detection. The incredible effort devoted to the development of highly sensitive detection methods able to finely detect biomarkers from biological samples with high specificity may pave the way to routinely employed AD diagnosis kits for clinical use.

Although nanotechnology is expected to have a huge impact on the development of “smart” drug delivery and theranostic devices against AD, a crucial gap still to be filled concerns the elucidation of its etiology, for which a great deal of effort is still required.

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