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Review

Extracellular vesicles in Alzheimer's disease: from pathology to therapeutic approaches

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Abstract

Alzheimer's disease is a progressive and fatal neurodegenerative disorder that starts many years before the onset of cognitive symptoms. Identifying novel biomarkers for Alzheimer's disease has the potential for patient risk stratification, early diagnosis, and disease monitoring in response to therapy. A novel class of biomarkers is extracellular vesicles given their sensitivity and specificity to specific diseases. In addition, extracellular vesicles can be used as novel biological therapeutics given their ability to efficiently and functionally deliver therapeutic cargo. This is critical given the huge unmet need for novel treatment strategies for Alzheimer's disease. This review summarizes and discusses the most recent findings in this field.

Key Words: Alzheimer's disease; brain; diagnostic; extracellular vesicles; isolation methods; microglia; neurodegenerative diseases; neuroinflammation; neurons; therapy

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by loss of memory and cognitive abilities and is the most common form of dementia. AD is a heterogeneous disorder with multiple phenotypes, which prevents an accurate early diagnosis and targeted interventions. The early diagnosis of AD is a major challenge due to the lack of reliable biomarkers and, unfortunately, AD also lacks effective treatment options.

Extracellular vesicles (EVs) are a heterogeneous group of membrane-bound structures including exosomes, microvesicles, and apoptotic bodies (van Niel et al., 2018). EVs contain proteins, lipids, and a wide variety of genetic materials, such as DNA, mRNA, and non-coding RNAs and are found in most biological fluids (Valadi et al., 2007). Moreover, EV cargo is protected from proteases and nucleases in the extracellular space. Current assessment and evaluation of AD use neuroimaging parameters together with biomarkers in the cerebrospinal fluid (CSF), which has to be obtained by an invasive procedure. EVs represent a promising alternative given they can be isolated from different sources using minimally or non-invasive techniques (Scarth et al., 2021). Given that brain-derived exosomes contain cargo from their original cells, and they can be isolated from peripheral blood and/or other biological fluids, they can therefore be used as biomarkers for the diagnosis, screening, prognosis, and monitoring of AD (Lugli et al., 2015; Chen et al., 2016; Arioz et al., 2021; Scarth et al., 2021). On the therapeutic side, there are no new drugs for AD which highlights the urgency for new therapeutic approaches. In recent years, EVs have also emerged as a novel therapeutic tool for neurodegenerative diseases given their ability to carry a therapeutic cargo to target cells. Hence, EVs may also represent an exciting new therapeutic intervention for AD, not only for treatment in established disease states but also for the prevention of neuronal damage and neuroinflammation in early disease states.

Search Strategy and Selection Criteria

In this narrative review, we used PubMed and Google Scholar to search articles published from 1998 to 2021 with the following keywords: extracellular vesicles, neurodegeneration, Alzheimer's, therapeutics, pathology, and biomarkers.

Extracellular Vesicles

EVs are a heterogeneous population of membrane-bound structures released by most cells into the extracellular space and can be found in most body fluids such as serum, plasma, urine, saliva, and cerebrospinal fluid (van Niel et al., 2018). EVs can be classified based on their size, origin markers, content, or source (**Table 1**).

Extracellular Vesicles Isolation Methods

EVs can be isolated from cell culture media or biological fluids including blood, urine, cerebrospinal fluid, tears, and saliva. Due to the complexity of biological fluids, current isolation methods isolate either exclusively exosomes or a mixture of EVs and other components. An overview of these methods can be found in **Table 2**.

Ultracentrifugation

Ultracentrifugation is the most commonly used method to isolate EVs. There are two types of ultracentrifugation methodologies: differential or density gradient. Differential centrifugation consists of sequential centrifugation steps: a 300 × *g* spin for 10 minutes followed by a 10,000 × *g* spin for 30 minutes to eliminate intact cells, dead cells, and cell debris. After depletion of cells and large apoptotic bodies by low-speed centrifugation, the EVs are pelleted in the final step at 100,000 × *g* for 70 minutes (Garcia-Contreras et al., 2017). Density gradient centrifugation is a combination of ultracentrifugation with a sucrose gradient to separate the EVs based on their density (Cvjetkovic et al., 2014).

Affinity-based capture

Affinity-based isolation enables the selective capture of specific EVs subpopulations using antibodies to specific membrane markers such as CD63, CD81, or CD9 (Kowal et al., 2016). For this reason, selecting a proper membrane marker is one of the most significant steps in these immunoassays. A bead-based affinity approach is a method that uses magnetic beads or latex beads for capturing and isolating EVs. Another affinity based-capture method is a lipid nanoprobe system, which labels the EVs with a labeling probe for magnetic enrichment using a capture probe (Bano et al., 2021).

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Table 1 | **Classification of extracellular vesicles**

Type of extracellular vesicle	Size (nm)	Biogenesis	Markers	Content
Exosomes	50–100	Exocytosis of multivesicular bodies	Tetraspanins: CD63, mRNA, CD9, CD81. TSG101, flotilin, Alix	mRNA, microRNA, non-coding RNAs, cytoplasmic and membrane proteins, including receptors and major histocompatibility molecules
Microvesicles	100–1000	Budding of plasma membrane	ARF6, VCAMP3, integrins, selectins, and CD40 ligand	mRNA, microRNA, non-coding RNAs, cytoplasmic and membrane proteins, including receptors
Apoptotic bodies	100–5000	Budding of the plasma membrane during apoptosis	Thrombospondin, C3b, Annexin V, phosphatidylserine	Nuclear fractions, cell organelles
Retrovirus-like particles	90–100	Direct budding of plasma membrane	Gag	Retroviral particles env, rec, and pol

Table 2 | **Extracellular vesicle isolation methods**

Method	Advantages	Disadvantages	Time required	References
Ultracentrifugation (Differential)	Isolation of large volumes	Inability to separate exosomes from microvesicles	8 h	Garcia-Contreras et al., 2017; Carnino et al., 2019
Ultracentrifugation (Density gradient)	Pure preparations	Inability to separate exosomes from microvesicles	20 h	Cvjetkovic et al., 2014; Carnino et al., 2019
Affinity based-capture		Co-purification of protein aggregates	Overnight	Kowal et al., 2016; Carnino et al., 2019
Filtration	Simple procedure	Protein contamination	2 h	Davies et al., 2012; Liang et al., 2017; Carnino et al., 2019
	No limitations on sample volume	Filter plugging (loss sample)		
Size exclusion chromatography	Pure preparations	Limitations on sample volume	1 mL/min + column	Carnino et al., 2019; Lane et al., 2019
	Preserves vesicle integrity	Specialized equipment and column		
	Prevents extracellular vesicle aggregation	Complexity		
Polymer precipitation	Simple procedure	Low purity and specificity, protein contamination	Overnight	Rider et al., 2016; Carnino et al., 2019
	Possibility of processing samples with a large volume			
Microfluidic technologies	Rapidness	Complexity of devices	1–2 h	Gholizadeh et al., 2017; Carnino et al., 2019
	Purity	Additional equipment		
	Efficiency	Cost		

Filtration
Filtration is used in combination with ultracentrifugation to isolate EVs using porous membranes that trap molecules or particles of a specific size, allowing smaller molecules and particles to flow through the membranous filter (Liang et al., 2017). Samples are then processed using a centrifugal filter, followed by recovery with a reverse spin. Although this method is simple and allows the processing of many samples, filter plugging might result in loss of sample, in addition, to sample contamination by proteins.

Size Exclusion Chromatography

Size Exclusion Chromatography separates EVs based on the size using chromatography or microfiltration. Column chromatography allows for sequential elution of EV size fractions from a single column (Lane et al., 2019). Once the column is set, the sample is loaded into the column and eluted samples are collected from the column. While this method allows relatively fast collection of EVs by size, there are sample volume limitations and complex equipment requirements.

Polymer precipitation
Polymers such as polyethylene glycol are used for the precipitation of EVs. There are several commercial polymer-based isolation methods such as ExoQuick, Invitrogen Total Exosome isolation reagent, or miRCURY™ Exosome Isolation Kit. These methods allow EV isolation from large samples with a high yield. However, contamination of EV pellets with non-exosomal materials remains a problem for polymer-precipitation methods and the polymer may interfere with the downstream analysis.

Microfluidic technologies
Microfluidic-based technologies can isolate EVs from biological fluid in an easily reproducible, convenient manner (Gholizadeh et al., 2017). Microfluidic devices allow the rapid and low-cost separation and detection of targets, and the devices can be simply made which lowers the production cost thereby making them commercially viable.

Extracellular Vesicles Characterization Methods

Electron microscopy
Morphological characterization is carried out using electron microscopy. Transmission electron microscopy is a standard technique used to characterize EV preparations. EVs analyzed by transmission electron microscopy often show a cup-shaped appearance, which is an artifact of the preparation procedure. Transmission electron microscopy can be combined with immunogold staining using gold conjugated antibodies to detect the presence of specific markers. Scanning electron microscopy is another approach to analyze EV morphology and structure, while atomic force microscopy is a type of scanning microscopy that allows imaging of the topology of EV surfaces with nanometer resolution.

Western blot assay and enzyme-linked immunosorbent protein assay
Western blot assay is a widely used method to characterize and detect EV proteins. Enzyme-linked immunosorbent protein assays (ELISA) are also used to quantify the number of exosomes based on the level of the exosome-associated proteins including CD9, CD63, and CD81. ELISA is more sensitive and provides more accurate protein quantification compared to western blots.

Flow Cytometry

Flow cytometry or imaging flow cytometry of EVs is performed by using beads coupled to antibodies that detect EV surface markers (Campos-Silva et al., 2019). The use of beads is due to the small size of EVs and the difficulty to detect EVs with most conventional flow cytometers. The challenge with flow cytometry of EVs relates to their small particle size and low refractive index, which makes them difficult to separate from background signals.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis uses light diffraction patterns to measure the size and the concentration of EVs. Direct size and concentration quantification can also be performed using the tunable resistive pulse sensing principle.

Direct Counting of Single Extracellular Vesicles

There are new techniques that allow the study of single EVs, thereby enabling the study of specific EV subpopulations. Super-resolution microscopy allows separating focal spots to visualize and directly count the number of single EVs as well as quantify their content (Lennon et al., 2019). Other than fluorescence-based EV visualization, there is an interferometric reflectance imaging method for single EVs. EVs can also be captured in an antibody array on a chip followed by the acquisition of interferometric images by a chip reader where the number and size of EVs can be acquired (Daaboul et al., 2016).

Extracellular Vesicles in the Pathogenesis of Alzheimer's Disease

EVs are membrane-bound structures that transport cargo between cells in the body (Mathieu et al., 2019), including the nervous system where they have been shown to be involved in several neurodegenerative diseases such as AD, Parkinson's, and amyotrophic lateral sclerosis. AD is characterized by the intracerebral accumulation of amyloid-β (Aβ) leading to Aβ plaque formation (Villemagne et al., 2018) and EVs isolated from the CSF have been shown to contain high levels of Aβ which is toxic to neurons cultured *in vitro* (Sardar Sinha et al., 2018) and in animal models *in vivo* (Elsherbini et al., 2020). One explanation for this is that EVs stimulate Aβ aggregation (Dinkins et al., 2014)

and Aβ aggregates are less efficiently cleared and degraded by astrocytes and microglia (Dinkins et al., 2016), thereby contributing to their dysregulation in AD. Tau aggregates are another major hallmark of AD (Villemagne et al., 2018) and EVs from the CSF of AD patients have also been shown to contain Tau and these can be transmitted to neurons where they induce Tau aggregation (Saman et al., 2012; Fiandaca et al., 2015; Wang et al., 2017). In neurodegenerative conditions, microglia have been shown to release higher levels of EVs in comparison to inactivated microglia (Clayton et al., 2021) and in AD these EVs contain Tau which can be propagated to neurons *in vivo* contributing to the progression of tauopathy (Asai et al., 2015; Clayton et al., 2021). Furthermore, the dysregulation of EV cargo in iPSCs neurons has been associated with the AD familial A246E mutant form of presenilin 1, which could participate in propagating tau pathology. EVs derived from the iPSCs carrying the A246E presenilin 1 mutation contain high levels of the amyloid precursor protein and are capable of inducing tau deposits in the mouse brain after *in vivo* injection (Podvin et al., 2021) (**Table 3**).

Extracellular Vesicles as Biomarkers of Alzheimer's Disease

Proteins cargo

Several proteins have been found to be altered in EVs derived from AD patient samples (Muraoka et al., 2020). Specifically, neuron-derived EVs in AD patients have been shown to have increased levels of alpha-globin, beta-globin, and delta-globin compared to healthy controls by liquid chromatography-tandem mass spectrometry proteomics analysis (Arioz et al., 2021). Furthermore, lysosomal proteins are also altered in neural-derived plasma EVs in preclinical AD samples. These autolysosomal proteins could distinguish preclinical AD patients from controls (Goetzl et al., 2015). Synaptic proteins such as NPTX2, AMPA4, NLGN1, and NRXN2α have also been reported to be decreased in neuron derived EVs from plasma of patients with AD. These proteins decreased significantly from the time of normal cognition in preclinical AD to the time of the development of AD dementia (Goetzl et al., 2018). Moreover, astrocyte-derived EVs have been shown to contain dysregulated protein cargo in AD samples such as β-site amyloid precursor protein-cleaving enzyme 1 and soluble amyloid precursor protein β (Goetzl et al., 2016). Microglia-derived EVs also have been shown to have altered protein cargo in AD mouse models and these appear to correlate with disease progression (Muraoka et al., 2021).

Lipid cargo

In addition to proteins, lipids have also been found to be dysregulated in EVs from AD samples (Su et al., 2021), which can be attributed to the lipid imbalance associated with AD. Human frontal cortex brain-derived EVs from AD patients are enriched in glycerophosphoethanolamine and polyunsaturated fatty acyl containing lipids (Su et al., 2021). Amyloid-induced astrocyte-derived EVs have been shown to be enriched in ceramide species, which might also contribute to AD (Wang et al., 2012).

miRNAs and mRNAs cargo

miRNAs in circulating EVs have been shown to serve as biomarkers for age-related cognitive decline. Several miRNAs are dysregulated in AD samples from plasma, serum, CSF, and brain tissue (Lugli et al., 2015; Cheng et al., 2020). Specific miRNAs such as miR-132 and miR-212 are dysregulated in neural EV samples from AD patients compared to controls (Cha et al., 2019), while miR-9-5p and miR-598 have also been found in EVs obtained from the CSF of AD patients (Saman et al., 2012) (**Table 4**).

Table 3 | **Studies showing EVs involved in Alzheimer's pathology**

Specimen type	Species	Primary EV isolation method	Findings	References
CSF and cell culture media	Human	Ultracentrifugation	Exosome-mediated secretion of phosphorylated tau	Saman et al., 2012
Brain and serum	Mouse	Ultracentrifugation	Exosomes stimulate the aggregation of $A\beta_{1-42}$ *in vitro* and *in vivo*	Dinkins et al., 2014
Brain and cell culture media	Mouse	Ultracentrifugation	Microglia-derived exosomes help propagate tau pathology in the mammalian brain	Asai et al., 2015
Cell culture media and serum	Mouse	Ultracentrifugation and Exoquick	Ceramide-enriched exosomes exacerbate AD-related brain pathology by promoting the aggregation of Aβ	Dinkins et al., 2016
CSF and cell culture	Human	Ultracentrifugation	Exosomes mediate the propagation of Tau aggregation between cells.	Wang et al., 2017
Plasma	Mouse	Exosome precipitation reagent	Plasma exosomes can decrease Aβ plaques and play a role in the pathological process of AD	Zheng et al., 2017
Brain (temporal neocortex)	Human	Ultracentrifugation	AD brains contain increased levels of Aβ oligomers and can act as vehicles for the neuron-to-neuron transfer of Aβ to recipient neurons in culture	Sardar Sinha et al., 2018
Brain	Mouse	Exoeasy Isolation	Exosomes enhance Aβ induced neurotoxicity *in vivo*	Elsherbini et al., 2020
iPSCs culture media	Human	ExoQuick-TC	AD familial A246E mutant form of presenilin 1 alters neuronal iPSCs EV cargo	Podvin et al., 2021
Brain	Mouse	Ultracentrifugation	Spread of tau in hippocampal GABAergic interneurons via brain-derived extracellular vesicles	Ruan et al., 2021

AD: Alzheimer's disease; Aβ: amyloid-β; CSF: cerebrospinal fluid; EVs: extracellular vesicles; iPSCs: induced pluripotent stem cells.

Table 4 | **Studies of findings on extracellular vesicles biomarkers in Alzheimer's disease**

Specimen type	Species	Biomarker	Primary EV isolation method	Findings	References
Astrocytes, cell culture	Human	Ceramide	Ultracentrifugation	Ceramide composition in amyloid-induced astrocytes is altered.	Wang et al., 2012
Serum	Human	miRNAs	Plasma/Serum exosomal RNA isolation kit	AD-specific 16-miRNA signature	Cheng et al., 2015
Blood and CSF	Human	Lysosomal proteins	Exoquick and immunoprecipitation	Autolysosomal proteins in neurally derived blood exosomes distinguish patients with AD from case controls	Goetzl et al., 2015
Plasma	Human	miRNAs	Ultracentrifugation	Screening of individual loci indicated that 20 miRNAs showed differential expression in AD	Lugli et al., 2015
Serum	Human	miRNAs	Total exosome isolation reagent	miRNA-135a,-193b, and-384 potential biomarkers for early AD diagnosis	Yang et al., 2018
Brain, iPSCs, CSF, and blood	Human	miRNAs	Exoquick and immunoprecipitation	miR-132 and miR-212 dysregulated in AD neural EVs	Cha et al., 2019
Plasma	Human	Protein	Ultracentrifugation	EV-bound Aβ measurement could better reflect PET imaging of brain amyloid plaques and differentiate various clinical groups.	Lim et al., 2019
Brain and serum	Human	Small RNA and miRNAs	Sucrose gradient and exosomal RNA isolation kit	BDEVs have differential RNA biotypes compared to a heterogeneous population of EVs and provide a better representation of the total brain.	Cheng et al., 2020
CSF	Human	Proteins	MagCapture exosome isolation kit	HSPA1A, NPEPPS, and PTGFRN were significantly increased in AD CSF EVs.	Muraoka et al., 2020
Blood	Human	Hemoglobin	Immunoprecipitation	Hemoglobin subunits and other peptides are altered in AD patients.	Arioz et al., 2021
Brain	Mouse	Proteins	Sucrose gradient, ultracentrifugation	Enrichment of Psen1, APP, and Itgax and reduction of Wdr61, Pmpca, Aldh1a2, Calu, Anp32b, Actn4, and Ndufv2	Muraoka et al., 2021
Human frontal cortices	Human	Lipids	Ultracentrifugation and density gradient	AD BDEVs have a unique lipid signature that distinguishes them from BDEVs of the CTL frontal cortex.	Su et al., 2021

AD: Alzheimer's disease; BDEVs: brain-derived extracellular vesicles; CSF: cerebrospinal fluid; CTL: age-matched controls; EVs: extracellular vesicles; iPSCs: induced pluripotent stem cells; PET: positron emission tomography; SEC: size exclusion chromatography.

Extracellular Vesicles as a Therapeutic Tool in Alzheimer's Disease

EVs can also function as a novel therapeutic tool for neurodegenerative diseases given they can efficiently target the brain and penetrate through the blood-brain barrier (BBB) (Alvarez-Erviti et al., 2011). Specifically, stem cell-derived EVs have been shown to have neuroprotective and immunomodulatory properties in neurodegenerative diseases (Niu et al., 2020; Garcia-Contreras and Thakor, 2021; Kim et al., 2021). In AD, the mechanism of Aβ degradation by glial cells is altered, including the production of proteases (such as neprilysin), which can hydrolyze Aβ at different cleavage sites. Previous studies have shown that administration of mesenchymal stem cell (MSCs) derived EVs overexpressing neprilysin reduced plaque deposition in AD mice models (Katsuda et al., 2013). Furthermore, MSC-derived EVs have shown to be neuroprotective in animal models of AD by protecting neurons against Aβ-induced oxidative stress and synaptic damage (de Godoy et al., 2018; Ma et al., 2020). This neuroprotection can be mediated by the delivery of MSC-EV specific cargo such as miR-29c-3p to neurons, which then inhibits BACE1 expression while activating the Wnt/β-catenin pathway (Sha et al., 2021). Neural stem cell-derived EVs have also been shown to have neuroprotective effects and can restore fear extinction memory consolidation and reduce anxiety-related behaviors (Apodaca et al., 2021). Finally, given that a lack of physical exercise contributes to several cerebral diseases, including AD, increasing EVs in the brain in response to physical exercise has been proposed as a potential therapeutic strategy (Zhang et al., 2021). New emerging engineering strategies are being developed to explore the specific targeting of EVs (Jang et al., 2021). EVs can be modified by manipulating their parent cells, with changes then subsequently incorporated into the secreted EVs for specific delivery/targeting (Dooley et al., 2021) (**Table 5**).

Table 5 | **Studies showing therapeutic applications of extracellular vesicles in Alzheimer's disease**

Mechanism	Findings	References
Dendritic-cell derived EVs as siRNA delivery vehicle	Exosome-mediated siRNA delivery by protein (62%) knockdown of BACE1, a therapeutic target in AD, in wild-type mice	Alvarez-Erviti et al., 2011
MSC neprilysin-bound exosomes	Administration of exosomes in the brain of AD mice causes a decrease in plaque deposition.	Katsuda et al., 2013
Sphingomyelinase inhibitor (GW4869)	Decreased EV levels are associated with less Aβ plaque deposition.	Dinkins et al., 2014
MSC-derived EVs	EVs protect neurons against AβO-induced oxidative stress and synapse damage.	de Godoy et al., 2018
ADSC-derived EVs	EVs alleviate neuronal damage and promote neurogenesis.	Ma et al., 2020
Neural stem cell-derived EVs	EVs restored fear extinction memory consolidation and reduced anxiety-related behaviors. EV treatment also significantly reduced dense core Aβ plaque accumulation and microglial activation.	Apodaca et al., 2021
MSC-derived EVs	EVs reduced Aβ expression and restored the expression of neuronal memory/synaptic plasticity-related genes in the cell model. Improvement in brain glucose metabolism and cognitive function in AD transgenic mice.	Chen et al., 2021
Bone marrow MSC-EVs	BM-MSC-EVs delivered miR-29c-3p to neurons remove to inhibit BACE1 expression and activate the Wnt/β-catenin pathway.	Sha et al., 2021
Exercise	Physical exercise increases EVs in the brain.	Zhang et al., 2021

ADSC: adipose derived stem cell; Aβ: amyloid-β; AβO: amyloid-β oligomer; BACE1: β-site amyloid precursor protein-cleaving enzyme 1; BM-MSC: bone marrow-derived mesenchymal stromal cell; EVs: extracellular vesicles; MSC: mesenchymal stem cell.

Conclusion

AD is an increasingly common form of dementia that worsens over time. While Aβ and tau are recognized as key factors in AD, many important details remain unknown. EVs could provide new insights into the underlying drivers of AD, with dysregulated EV cargo possibly representing an underappreciated driver of AD pathology. In addition, some of these EV cargos could also be reflective on the stage of AD and thus be used as biomarkers. Existing efforts in the field are trying to investigate how to analyze cell-type-specific EV content. Current biomarkers for AD are obtained from the CSF, which is obtained in an invasive manner and sometimes is not able to differentiate AD from other types of dementia. In contrast, EVs could be obtained from blood in a minimally invasive manner with studies suggesting they could even reflect the disease stage of AD (Goetzl et al., 2015; Lugli et al., 2015). Brain-derived EVs are thought to be present in the circulation given that neuroinflammatory responses and pro-inflammatory cytokines promote the breakdown of the BBB. However, given their low concentrations, the development of highly sensitive methods for EV detection and isolation is needed.

Existing AD treatments have a low efficacy partially due to the difficulties in crossing the BBB. However, EV-based therapies could represent a more efficient, specific, and functional delivery system. EVs have the ability to cross the BBB by interacting with the endothelial cells (the first line of defense in the brain). There is an urgent unmet need to develop new treatments to delay or prevent AD. Promising results using different types of EVs have been obtained, and these include reducing Aβ plaque deposition, oxidative stress, synaptic damage, and/or microglial activation (Katsuda et al., 2013; de Godoy et al., 2018; Apodaca et al., 2021). New engineering strategies to over-express brain cell-specific markers into EVs could develop an even more efficient and targeted drug delivery system for neurodegenerative diseases such as AD.

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