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Traversal of the Blood Brain Barrier with Nanotechnology for the Treatment of Alzheimer's Disease

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ABSTRACT

Alzheimer's Disease is a neurodegenerative disorder on the rise, expected to become a global public health crisis in the future as prevalence increases. The primary symptoms are memory impairment, declining cognition, mood disturbances and progressive degeneration ultimately leading to fatality. Very little is known about the etiology of Alzheimer's Disease, however the cause of the disease is neurobiological, characterized by amyloid β plaque accumulation and aggregation, tau protein hyperphosphorylation, subsequent oxidative stress and neuroinflammation. Much of the current research is limited by the inability of therapies to cross the blood brain barrier, which discriminates based on surface area and particle size, resulting in limited treatment options.

Treatment limitations have prompted further research into the field of nano-therapies that can cross the blood brain barrier to treat the suspected neurobiological causes of Alzheimer's Disease. In this paper, research using black phosphorus nanosheets and polymeric nanoparticles including PEG, PAMAM and chitosan are explored and summarized for their abilities to cross the blood brain barrier. Through their use as drug delivery agents, or via their innate molecule characteristics, there is a wealth of research currently undertaken to explore their use for this necessary purpose. Additionally, further focus is shed in this article about future avenues in light of the results of the research conducted.

Keywords

Alzheimer's Disease, Traversal of the Blood Brain Barrier, Nanotechnology, Nano drug delivery systems, Blood brain barrier (BBB), Nanosheets, Polymeric nanoparticles, siRNA encapsulated in polymeric nanoparticles.

Introduction

Alzheimer's Disease (AD) is a degenerative disorder affecting the central nervous system (CNS). Often accompanied by amyloid plaques and tau proteins, Alzheimer's Disease causes neuron death as well as atrophy of brain matter. This can affect the memory initially, but progresses to an inability to manage daily living, as well as emotional and mental changes, eventually leading to death [1]. It is estimated that by 2050, more than 152 million people worldwide will be afflicted with Alzheimer's Disease [2]. Despite affecting so many people currently, treatment options for Alzheimer's Disease are sparse, with the last drug to be approved by the FDA being memantine in 2003 [3]. Therefore, there exists a potential public health interest for there to be better treatment options for affected patients.

Alzheimer's Disease is complex, and the causes are poorly understood, making it difficult to prevent or treat. Scheltons et al. [4] characterize AD as a group of specialized pathophysiological changes which ultimately manifest in complex changes in a person's behavior, mood and mental state. The initial preclinical stage is cellular, where changes in neurons, microglia and astroglia occur before progressing to the clinical stage, where cognitive impairment manifests in the affected person. Inflammation, changes in the blood brain barrier, aging and glymphatic system changes are postulated to contribute to the buildup of Amyloid β plaques [4]. Continuous buildup of these plaques are hypothesized

to cause the upregulation and accumulation of tau protein via hyperphosphorylation into neurofibrillary tangles (NFT) [5]. Long et al. also postulates that aggregation of tau protein may be the primary reason behind neurodegeneration in AD and may also act in a manner similar to prion diseases, where misfolded proteins can self-propagate.

The blood brain barrier (BBB) exists as a highly discriminatory obstacle to prevent foreign pathogens and substances from reaching the brain and is composed of tightly joined endothelial cells with tight junctions, adherents junctions, pericytes and astrocyte end feet. The endothelial cells act as the core backbone of the blood brain barrier and provide a structure in which other components can attach to aid in protection. Tight junctions help in keeping the permeability of the BBB low, ensuring low diffusion across the membrane. Adherens junctions provide structural stability to the BBB through linkage to the cytoskeleton [6]. Astrocytes work within the BBB via their end-feet, which link them to the endothelial cells and pericytes [7]. They tackle neuroinflammation, clear neurotransmitters and assist pericytes in regulating the BBB [8]. Pericytes serve their role in the BBB by communicating with endothelial cells, with which they cover a vast majority. They play a vital role in the maintenance of the BBB, often adjusting the components in response to changes along the barrier. Additionally, pericytes work with astrocytes and endothelial cells in the development of vasculature, often interfacing directly with blood vessels [6]. Due to the tight nature of the endothelial cells and the work of tight junctions, astrocytes and pericytes, diffusion and molecule exchange is difficult unless expressly permitted by the BBB.

Many diseases affecting the central nervous system are able to do so when the blood brain barrier is disrupted. Bowman et al. [9] demonstrated that the damage to the BBB via neuroinflammation can lead to a progression of neurodegenerative disorders like Alzheimer's Disease. Additionally, Bowman et al. also found that endothelial cells in damaged blood brain barriers can leak vital cell components that help upkeep the BBB. Han et al. [10] discusses dysfunction in the BBB due to loss of tight junctions and degeneration of pericytes and endothelial cells. Han et al. also describes a paradoxical phenomenon in which damaged cells in the BBB can lead to improved drug delivery across the barrier but result in poorer distribution. Zenaro et al. [11] notes previous studies that have found changes in tight junction proteins that occurred due to amyloid plaque buildup, ultimately leading to disruption of the BBB in patients with AD. It was also found that levels of GLUT1, a glucose transporter, were decreased in the neuro-vasculature of patients with Alzheimer's Disease [12]. This alteration leads to a lack of nutrients that are provided to the brain of patients with AD [11]. Zlokovich et al. [13] found that impairments in endothelial cells regarding amyloid beta peptide transporters could contribute to the accumulation of amyloid beta plaques. Zenaro et al. discuss impairments in pericyte function, whereby pericyte loss correlated with higher levels of BBB impairment. In summation, little changes in the fabric of the BBB can cascade into further degeneration, accelerating disease in short periods of time.

Penetration of the blood brain barrier is extraordinarily difficult, current therapies for treatment of Alzheimer's Disease must meet specific criteria and must be transported via passive transcytosis. This transport across the endothelial layer can take place across two possible avenues, transcellular and paracellular. Wu et al. [6] elaborates on the transcellular pathway as the desirable pathway for transportation of carrier-mediated compounds, as they can be transported through the cell membranes. Due to the phospholipid bilayer of the cell membranes, carriers that are lipophilic are transported this way [6]. Transporting molecules through the paracellular space is difficult due to the presence of tight junctions, which can exclude molecules due to size, charge, and molecular weight [14]. These restrictions can make it difficult for small molecules to cross the BBB. Pardridge [3] outlines the same factors, noting that molecular weight, and polar functional groups are factors that eliminate smallest molecules from reaching the brain. Memantine, the last drug to be approved by the FDA for AD, is under 400Da, and forms less than 7 hydrogen bonds, which allows it to cross the BBB.

Nanotechnologies are considered to be technologies that are within the nano-scale. As materials reach the nano-scale, their properties become quite different due to the increase in surface area relative to volume ratio, which provides additional quantum properties. Other properties such as melting temperatures, charge, and magnetic properties can vary for nanomaterials with respect to their bulk materials without a change in chemical composition [15]. Their surface to volume ratio also provides nanoscale molecules with unique advantages for drug delivery systems, in which there can be increased specificity of ligand targeting, decreased dosage needed for efficacy, as well as less unintended side effects for nano-therapies. Another key feature of nanotechnologies is the ability to modify them for specific use. Nanoparticles can be modified for use in therapies due to their unique size, shape, surface charge, ability to functionalize their surfaces, ability for targeting and release, as well as agglomeration abilities [16]. Understanding Alzheimer's Disease, the function of the blood brain barrier, how the BBB becomes disrupted, as well as current shortcomings in penetrating the BBB to deliver therapies illustrate the reality of trying to treat AD: more treatment options are needed to help affected patients. To this end, further research with nanotechnology could yield fruitful results. Several nanotechnologies have the potential to help in penetrating the BBB, including black phosphorus (BP) nanosheets, polymeric nanoparticles, and siRNA encapsulated in polymeric nanoparticles. These technologies have demonstrated effectiveness in penetrating the BBB, as well as have showed treatment efficacy in studies using AD analogous mice models.

Nano Drug Delivery Systems

Nanosheets have an advantage due to their large surface area, notable even among other nanotechnologies, which allows for the loading and delivery of therapeutic agents due to many anchoring sites and large wells for therapeutic agents. In regard to biocompatibility with biological systems and the human body, the composition of

nanosheets is exceedingly important to ensure proper elimination and the avoidance of accumulation in the human body, which is particular risk regarding nanotechnology [17]. Qian et al. notes that phosphorus is essential for the functioning of the human body, due to which there exist many mechanisms for metabolism and elimination. Of the many forms of phosphorus, black phosphorus (BP) is the most biocompatible due to its stability. Additionally, BP has an additional advantage, wherein bulk crystals of BP can be engineered into single layer nanosheets with a multitude of anchoring points for several kinds of therapies [17].

In addition to black phosphorus' role in nanosheets with the ability to load therapeutic agents, Chen et al. [18] note that BP nanosheets have other unique properties that have the potential to be helpful in the treatment of neurodegenerative diseases such as AD. Phosphorus in the BP nanosheets have a strong affinity for metal ions, the latter of which is involved in the degeneration of neurons due to Cu^{2+} catalyzed reactive oxygen species (ROS). Chen et al. demonstrates that BP nanosheets can act as an effective captor for Cu^{2+}, thereby reducing ROS formation that leads to neuronal apoptosis. Chen et al. also demonstrated that near infrared irradiation (NIR) can increase the ability of BP nanosheets, via photothermal effect, to penetrate the blood brain barrier of mice, without causing significant damage via hyperthermia to the brains of the mice.

BP Nanosheets for Waste Removal of Tau Proteins

Ma et al. [19] conducted a study using BP nanosheets for the treatment of Alzheimer's Disease, where the nanosheets acted as a waste vehicle to remove accumulated tau protein from the brain across the BBB. Discussed earlier by Long et al. [5], tau protein accumulation appears to occur alongside, and perhaps due to, the proliferation of amyloid beta plaques. The accumulation of these tau proteins can contribute to and may even be a primary force behind AD related neurodegeneration. Ma et al. seek to understand if tau protein can cross the BBB to be removed from mice brains using BP nanosheets. Initially, the BP nanosheets were modified to be fitted with PEG (BP-PEG), then characterized ensure that the nanosheets met the specific size, thickness and energy requirements as well as to ensure proper preparation of the nanosheets. X-ray photoelectron spectroscopy (XPS) showed the ability of the phosphorus atoms on the BP surface to be readily oxidized, noting proper preparation [19]. With the lack of clinical studies taking place, much of current nanotechnological research must use mouse analogues to understand and demonstrate efficacy. Baker et al. [20] conducted a study using wild type mice where okadaic acid (OA) was injected in the brains of wild type mice. It was found that injecting OA into the brain can induce tau protein hyperphosphorylation by inhibiting PP2A, which has the primary function of dephosphorylating tau protein. This chain leads to tau protein propagation, which can spread across the brain in a similar manner to AD [20]. Ma et al. [19] use this mechanism to induce tau hyperphosphorylation for the purposes of their study. The researchers confirmed that tau hyperphosphorylation took place after injection of OA into the mice brain, by measuring levels of neurofibrillary tangles in the cortex, along with behavioral tests to confirm the OA mice were affected. Several behavioral tests, including the 5-choice serial reaction time (5-CSRT), paired associate learning (PAL), and open field tests, were conducted on the OA mice. The results of the behavioral tests showed that OA mice performed worse than control group mice, indicating that the OA had the intended effect. Ma et al. also conducted a safety evaluation with intravenous administration of BP-PEG, measuring cellular activity and confirming that BP-PEG did not cause noteworthy hemolysis. Additionally, it was found that the nanosheets accumulated in the liver and kidneys, where they could be easier metabolized and cleared. Ma et al. also delineated the effects of the BP and BP-PEG on the tau proteins using trans electron microscope, as well as performing molecular dynamics (MD) simulation. It was found that BP-PEG was able to inhibit tau protein aggregation, and cause fragmentation of the tau protein, while MD confirmed tau protein binding to the BP nanosheets [19].

Ma et al. [19] conducted several behavioral tests to gauge the effectiveness of the ability of BP-PEG to transport tau across the BBB. The 5-CSRT test showed that OA mice intravenously administered with BP-PEG were comparable to the control group mice, but also that BP-PEG could rescue functions that were impaired by rampant tau hyperphosphorylation. The PAL test demonstrated that BP-PEG mice performed similarly to the control group mice, and confirmed that impairment in cognition could be restored after treatment with BP-PEG. When conducting the open field test, Ma et al. found that the OA mice were impaired, often traveling slower and more disorganized than mice in the control group. However, mice in the BP-PEG group showed improvement, with less time spent in the open field. Lastly, the Morris water maze (MWM) test and the nest construction behavior test both confirmed that the OA group performed the worst out of the three groups, but BP-PEG mice performed similarly on this test as control group mice. This further confirms the potential of BP-PEG to rescue cognitive functions that were impaired by tau hyperphosphorylation. The findings of Ma et al. illustrate the versatility of BP nanosheets as a carrier for tau protein across the BBB.

BP Nanosheets as a Vehicle for Anti-Inflammatory Therapy

Ma et al. [19] demonstrated the ability of BP nanosheets to act as a waste removal molecule, by transporting tau protein across the BBB and out of the brain of OA mice. In the same vein, Xie et al. [21] demonstrates that BP nanosheets can be used as a vehicle to carry antioxidants across the BBB to combat inflammation caused by reactive oxygen species (ROS) in addition to tau hyperphosphorylation thought to be caused by inflammation. The anti-inflammatory agent used by Xie et al. is known commonly as methylene blue (MB), but its chemical name is hydromethylthionine mesylate (HMTM). Methylene blue is thought to have many functions, but its most relevant function for nanotechnological use is as an anti-inflammatory agent. Gureev et al. [22] highlights several studies that have shown that MB decreases levels of TNF-α, a multi-functional cytokine involved in inflammatory responses. The downregulation of TNF-α causes a downstream effect on NF-κB, which controls several genes

involved in the inflammatory response. Gureev et al. also note that MB inhibits NF-κB, along with many other factors responsible for inflammation, which in combination with downregulation from TNF-α inhibition, illustrate the effect of MB on the inflammatory process. A Phase 3 double blind randomized clinical study (LUCIDITY) demonstrated that treatment with HMTM were able to elicit a statistically significant reduction in neurofilament light chain (NfL) plasma levels, which are a well-known biomarker for neurodegeneration associated with AD [23].

Xie et al. [21] prepared the nanosheets using a method previously used by Cheng et al. [24], through liquid-phase exfoliation. After the BP nanosheets were prepared, they were mixed with MB in ultrapure, deoxygenated water and stirred continuously. Centrifuging allowed the material to precipitate out, after which it was washed to decontaminate the material and receive BP-MB. Xie et al. then characterized the BP-MB via TEM, Atomic Force Microscopy (AFM), after which XPS, Fourier transform infrared spectrometer (FTIR), and ultraviolet-visible (UV-Vis) spectroscopy all confirmed BP-MB synthesis. To ascertain and confirm the safety profile of the therapies used in the study, Xie et al. conducted an assessment of biocompatibility. This included organ index analysis, as well as histology analysis via H&E staining of organ tissue. It was determined that there were no organ damage caused by the therapeutic agents, nor any organ dysfunction in the liver and kidneys, which was determined by using biochemical indexes [21].

To ensure BP-MP crossing of the BBB, Xie et al. [21] conducted several in-vitro tests using bEnd.3 cells. bEnd.3 cells have murine lineage and are typically used in *in vitro* studies to study the BBB [25]. Sun et al. also notes that the bEnd.3 cells exhibit phenotypic markers characteristic of brain endothelial cells. This corroborated a study previously done by Ku et al. [26], investigating bEnd.3 cells for the study of ischemia in the brain. Xie et al. [21] also use transepithelial electrical resistance (TEER) to measure the resistance of the bEnd.3 cells, which must have registered above 200Ω/cm^2 to be used. Using BP to interface with Cyanine 5 (Cy5) to help track the BP, Xie et al. were able to show that the black phosphorus was taken up by endothelial cells with the help of NIR [21].

In a similar manner as Ma et al., Xie et al. [19,21] also use OA to simulate AD by injecting the OA solution into the central compartment of the brain. These OA mice were randomized into treatment groups consisting of control group mice with no treatment, PBS (phosphate buffered saline), lone MB, BP-MB, and BP-MB with NIR. After randomization and administration, the mice were subject to behavioral tests to gauge the efficacy of each of the treatments. When conducting the Morris Water Maze (MWM), Xie et al. found that compared to the control group, OA mice treated with PBS performed the worst out of all the groups, demonstrating memory impairment. The groups treated with MB, BP-MB and BP-MB + NIR showed an ability to learn from past errors in the maze after re-training, as well as faster locomotor abilities, which the OA mice did not show. After the MWM, Xie et al. exposed the mice to an open field test to analyze the movement abilities of each treatment group. The OA group was deemed to be the least curious about the open field, with minor locomotion, but the mice from the other treatment groups demonstrated higher curiosity by exploring the open field, as well as increased locomotor capability [21].

Xie et al. [21] also conducted the novel object recognition test, which corroborated the findings from the MWM and the open field test. The OA group scored lower than the other groups on accuracy with a higher rate of errors. The BP-MB + NIR group performed the best out of all the groups, with similar results as the control group. Lastly, the paired associate (PAL) test was conducted, again confirming the earlier behavioral tests of Xie et al. The OA group performed the worst of all the groups, while the BP-MB+NIR group performed notably better than the other groups. The behavioral tests conducted by Xie et al. indicate that the BP-MB combination can be effective, while the BP-MB + NIR group showed that NIR can be an important step in ensuring BBB permeability for BP-MB. Pathological studies conducted by Xie et al. showed that the BP-MB + NIR treatment was able to reverse the impairment caused by the OA. Additionally, it was found that this combination was able to inhibit tau aggregation, prevent neuronal mitochondria malfunction via ROS scavenging, and rescue other downstream effects that tau has on neuroinflammation [21].

The study conducted by Xie et al. indicate an important use of BP nanosheets for the potential treatment of AD. The use of methylene blue combined with BP nanosheets as a delivery vehicle and treatment of NIR was proven to be a successful combination in treating OA mice. Several *in vitro* and *in vivo* behavioral tests demonstrated the ability of BP-MB to reverse neuroinflammation and subsequent neuroapoptosis. Lastly, Xie et al. were able to demonstrate that the therapies were safe when conducting a hematological analysis of the mice in each therapeutic group. Biochemical indexes verified that no organ impairment took place.

Using nanoparticles to Deliver siRNA across the BBB

A key feature of the research of Ma et al. and Xie et al. was the rescue of functions that were lost due to the accumulation of tau protein and ROS. Bazzari et al. [27] discuss a hypothetical pathway leading to amyloid β proliferation. This pathway focuses on a specific enzyme known as BACE1, which is responsible for the cleavage of amyloid precursor protein (APP). APP is responsible for a multitude of developmental neurobiological functions. APP is usually cleaved by the α-secretase and γ-secretase enzymes, which give rise to soluble APP-α molecules. Cleavage by BACE1, and then γ-secretase results in non-soluble amyloid β isoforms ($A\beta_{42}$). Bazzari et al. note that this isoform is prone to amalgamation, leading to the formation of amyloid β plaques. Zhou et al. [2] seek to distort this chain using nanoparticle attached siRNA traverse the BBB to silence the BACE1 gene. Using glucose transporter-1 (GLUT1) recycling, they developed a delivery system built on nanotechnology to improve BBB penetration in mice.

Small interfering RNA (siRNA) are bits of RNA with the ability to silence genes with increased specificity and effectiveness [28].

Part of a mechanism noted as RNA interference, siRNAs have a high degree of potential for therapeutic uses. However, promising siRNAs are, they have disadvantages that affect their efficacy, mainly stability issues and susceptibility to various enzymes that are able to break down the RNA [29]. Zhou et al. [2] overcome this vulnerability with the use of nanotechnology, in the form of Gal-NP. Gal-NP was complexed to siRNA in a process they describe as "triple interaction", where PEG-P(GuF), Gal-PEG-P (Gu) undergo self-assembly and combination with siRNA to form Gal-NP@ siRNA. The attachment of galactose to the nanoparticle is the basis for the transport across the BBB. Glycosylation using galactose allows for the nanoparticle to attach to GLUT1, which is able to pass through the endothelial cells of the BBB [2].

Confirming this first in *in vitro* studies, Zhou et al. used flow cytometry and confocal imaging to show that Neuro-2a cells take up the siRNA nanoparticles. In addition, Zhou et al. were able to demonstrate GLUT1 as a primary pathway for endocytosis, as well as confirm BACE1 gene silencing and protein downregulation. It was previously found by Mooradian et al. [12] that GLUT1 is reduced in AD patients. This fact poses an interesting dilemma for the mechanism of the work of Zhou et al. However, they overcome this mechanism by inducing a fasting state in mice, which allows for the overexpression of Glut1 in mouse models, which allows Gal-NP@siRNA to bind to Glut1 and pass through the BBB. Using Cy5 for tracking, they show that fluorinating Gal-NP@ siRNA allows for longer circulation and elimination times *in vivo*.

When evaluating the efficacy of Gal-NP@siRNA, Zhou et al. use APP/PS1 transgenic mice, instead of using OA damaged mice. Mice with APP/PS1 gene mutation have been shown to develop larger amounts of amyloid deposits, as well as loss of synapses when compared to other mouse models. Additionally, APP/PS1 mice present a good experimental model for BACE1 silencing. Similar to other studies where mouse models are used, Zhou et al. use behavioral tests to assess the impact of the nano-therapy on the mice. The NOR, MWM and nest building tests were used to measure the cognitive and general functional capability of the affected mice when compared to wild type. The mice were randomized into several groups, wild type treated with PBS used as controls (WT), APP/PS1 mice treated with PBS, APP/PS1 mice treated with siRNA nanoparticles but without glycosylation using galactose, APP/PS1 mice treated with glycosylated nanoparticles, but with scrambled siRNA, and APP/PS1 mice treated with GAL-NP@siRNA [2].

Using nesting data, Zhou et al. demonstrated that the Gal-NP@ siRNA mice received similar nesting scores as wild type mice, when compared to the other APP/PS1 groups. The NOR test showed the effects of the Gal-NP@siRNA treatments when comparing before and after data of the nano-therapy. Before treatment, the APP/PS1 mice with PBS treatment scored lower, with little interest in exploring novel objects, with testing and treatment with Gal-NP@siRNA, the APP/PS1 mice showed increased interest in exploration, as well as nearing the performance of wild type mice. The Morris Water Maze did not yield results showing the efficacy of siRNA knockout nano-therapies, as all groups showed similar results.

After the behavioral experiments were conducted, the brain tissues of the mice from the various groups were analyzed to understand the effects of the Gal-NP@siRNA treatment on amyloid precursor protein and the downstream effects of amyloid deposition. The mice under treatment for Gal-NP@siBACE1 experienced lower levels of BACE1 in the hippocampus and cortex when compared to the other groups, correlating with the behavioral tests administered by the researchers. Coupled with this finding, amyloid plaque development was significantly decreased in the hippocampus and cortex in these mice as well. Consistent with that logic, the mice from the other groups that were not treated with Gal-NP@siBACE1 were found to have elevated levels of Aβ plaque deposits. As previously characterized by Long et al. [5], the neurofibrillary tangles (NFT) of accumulated tau protein are also a key feature of AD, which works in synergy with the Aβ plaques in the symptoms of AD. Although not the primary focus of this study, when analyzed by Zhou et al., tau levels in mice treated with Gal-NP@siBACE1 were lower in the hippocampus and cortex than those in the control mice. Another key aspect of the effects of the knockout gene therapy applied by Zhou et al., is that alteration in BACE1 activities and subsequent absence can negatively affect the process of remyelination. When checking key markers for monitoring remyelination, Zhou et al. noted that expression of important markers for remyelination were decreased in the control group mice with PBS treatment, but the same biomarker levels were similar as seen in wild type mice.

Zhou et al. demonstrated the application of siRNA knockout gene therapy to prevent the formation of amyloid β formation. They were able to affect statistically significant results when treating the symptoms in mice. In addition to efficacy, they were able to demonstrate safety by measuring important hematological and general chemistry parameters. Examinations of liver enzymes, red blood cell and white blood cell counts did not show any difference between the control group mice and the siRNA mice. There were also no differences in eating habits, or body weight change in the mice along with no necrosis or apoptosis in major organs after 10 cycles.

Regulation of Microglia with PAMAM Nanoparticles

The studies of Ma et al. [19], Xie et al. [21] and Zhou et al. [2] all have a focus in the prevention and control of early symptoms of AD through intervention in the proliferation of amyloid β plaques or tau hyperphosphorylation. Zhong et al. [30] explore the possibilities of treatment with more advanced AD symptoms in APP/PS1 mice by regulating microglia with Prussian blue-PAMAM-Angiopep-2 (PPA) nanoparticles. In a method previously used by Zhou et al., Zhong et al. use APP/PS1 mice, which have previously been characterized as having elevated levels of amyloid β plaques, as well as larger amounts of synaptic loss. Prussian Blue is thought to have reactive oxygen scavenging abilities due to its specificity in binding hydroxyl radical groups and its ability to mimic three enzymes with antioxidant abilities, peroxidase, catalase, and

superoxide dismutase [31]. PAMAM is a dendrimer with capacities for drug delivery, molecular encapsulation and gene therapies [32]. Angiopep-2 is a ligand that binds to the target receptor, low-density lipoprotein receptor-related protein-1 (LPR-1), on the surface of endothelial cells of the blood brain barrier. Microglia are immune cells of the central nervous system and function in a phagocytic role to clear the CNS of debris, with an important role in defense of pathogens, injury responses, and tissue maintenance. Hanson et al. [33] describe the ability of microglia to modulate synaptic loss, as well as exacerbate tau hyperphosphorylation. Microglia action in inflammatory mediation is thought to have a role in secretion of toxic factors that can injure neurons. This key process can be distorted during the early stages of AD, and accelerate the progression into symptomatic AD. Additionally, damaged microglia can become overactive, leading to impairment of functional microglia. Zhong et al. seek to intervene in the process of microglia malfunction and restore their original function with PPA nanoparticles.

Synthesis of the PPA nanoparticles took place in two separate steps; first, Zhong et al. [30] prepared the Prussian Blue-PAMAM particles (PP). First the PAMAM, purchased from a supplier, was dissolved in de-ionized water and then mixed with another solution of $FeCl_3$ and Gd $(NO_3)_3$). Next this mixture was added to another solution containing $K_4Fe(CN)_6$ and vigorously stirred. This was then centrifuged and purified via resuspension with de-ionized water. Next, to make the PPA nanoparticles, the PP nanoparticles were functionalized with angiopep-2 via a series of reactions. To confirm that the PPA nanoparticles were of the appropriate purity, structural and topographical analysis was performed. Scanning electron microscopy and transmission electron microscopy showed that the nanoparticles were spherical, with a size of ~49 nanometers, confirmed by dynamic light scattering detection. The infrared spectrum analysis showed a characteristic peak corresponding to a cyano group, which indicated that Angiopep-2 had successfully attached to the PPA nanoparticle surface. To measure the extent to which the PPA nanoparticles had functionality in ROS scavenging, peroxidase and catalase activities were measured when in presence of the PPA nanoparticles. Results from analytical tests for activities of both catalase and peroxidase indicated that the PPA nanoparticles increased the activity of both enzymes.

To ensure that the PPA nanoparticles were not cytotoxic, cytocompatibility assays were conducted *in vitro* and *in vivo*. Various concentrations of the PPA nanoparticles were assayed and determined to have no major effects on cell viability with lower concentrations. *In vivo* safety experiments were performed by injection of PPA nanoparticle solution into mice, where histological analysis noted that there were no significant impacts on internal organ tissues when compared to untreated mice. An *in-vitro* efficacy analysis was conducted by inspecting the effects of amyloid β toxicity on BV-2 cells [30]. Henn et al. [34] note that BV-2 cells are a suitable replacement for primary microglia in *in-vitro* settings, acting as a model for neuroinflammation. When analyzing the effects on BV-2 cells, Zhong et al. found that the exposure to amyloid β severely decreased the BV-2 cell effectiveness. They also found that PPA restored the viability of the amyloid β exposed BV-2 cells, and had a protective effect against the amyloid β. To confirm the effectiveness of the angiopep-2 to assist the PPA nanoparticles in crossing the BBB, mice brain capillary endothelial cells were used as a BBB model. TEER was measured until they reached levels characteristic of the BBB *in vivo*. When adding the PPA nanoparticles, the PPA nanoparticles showed higher penetration than the PP nanoparticles. When injected into the mice tails, histological analysis of brain tissue showed permeability across the BBB in APP/PS1 mice [30].

To evaluate the function of the PPA nanoparticles to affect the microglia *in vivo*, biomarkers were used to track membrane ruffling of microglia in APP/PS1 mice. Zhong et al. describe membrane ruffling as responsible for the changes in adequately functioning microglia to become hyperactive. By tracking the membrane ruffling, the researchers were able to demonstrate the effectiveness of the PPA nanoparticles and their suppression of the transformation to hyperactivity. Brain tissue histological analysis demonstrated that amyloid β buildup was significantly lower in the APP/PS1 mice treated with PPA nanoparticles. To confirm the overall impact of the PPA nanoparticles on the microglia, the researchers looked to investigate the function of the neurons and how they would be impacted by the rescue of the microglia. They used positron emission tomography (PET) to assess glucose metabolism in the brains of the treated mice, which has a positive correlation with neuronal function. In coordination, Zhong et al. also conducted behavioral tests to gauge the learning and memory of the treated mice vs the control group mice. They found no significant differences in the locomotor portion of the MWM test, but did note that the time taken to find the invisible platform was progressively decreased in the PPA nanoparticle mice, indicating higher degree of memory function. There was also no significant difference between the WT mice and the PPA nanoparticle mice, showing that spatial memory functions could be rescued with the PPA nanoparticles.

Zhong et al. show a conclusive method of bypassing the blood brain barrier and targeting early mechanisms thought to possibly cause Alzheimer's disease. They were able to show conclusive data that illustrated the ability of the PPA nanoparticles to traverse the BBB, as well as positively act on hyperactive microglia. Additionally, they were able to demonstrate that the PPA nanoparticle therapy could also help prevent the formation of amyloid β plaques as well as rescue neuronal functions in APP/PS1 mice. Doing so also enabled them to evaluate behavioral changes and confirm the rescue of memory capacities such that they were similar to wild type mice.

Binding of Amyloid β Plaques with BBB Permeable Chitosan Oligosaccharides

Chitosan is polymeric nanoparticle made of polysaccharides and derived from crustacean shells [35]. It has a multitude of properties that make it ideal for use in biopharmaceutical applications, such as its ability to be biocompatible, nontoxic, nonimmunogenic, anti-inflammatory, anticoagulant and can be degraded easily by

enzymes [36]. Zhu et al. [37] also note chitosan's ability to be neuroprotective due to its anti-inflammatory nature. Recent studies suggest that chitosan inhibits amyloid β aggregative plaques through isolation of individual peptides in substantial amounts and limiting their aggregation [38]. Zhu et al. [37] conduct a study investigating whether chitosan oligosaccharides (COS) are able to cross the blood brain barrier and bind with amyloid β plaques for the treatment of Alzheimer's Disease.

First to confirm that the COS were corrected synthesized, the researchers characterized them with NMR spectroscopy and dynamic light scattering and confirmed correct size and weight. To confirm the permeation of the chitosan oligosaccharides across the BBB, Zhu et al. use an *in-vitro* and *in-vivo* model. Similar to Xie et al. [21], the researchers use bEnd.3 cells as a model for the blood brain barrier. First, Zhu et al. confirm the ability of the bEnd.3 cells to act as a barrier with the ability to discern molecule sizes by measuring the permeability coefficients. They were able to show that the chitosan nanoparticles penetrated the in-vitro model composed of bEnd.3 cells. It was also not clear if GLUT1 was necessary for the COS to penetrate the BBB, therefore testing with inhibition of GLUT1 was conducted and shown to be necessary. To demonstrate penetration *in vivo*, COS was first labeled with Cyanine 7 (Cy7) and co-administered with Cy7-NHS, the latter acting as a control. Fluorescence intensity imaging and subsequent neuro-histological analysis revealed that the COS nanoparticles were able to penetrate the BBB *in-vivo* [37].

Confirmation of the efficacy of the COS occurred by examination with ThT fluorescence assay and transmission electron microscopy. The group of mice treated with COS showed statistically significant results, with a decrease in amyloid β NFT when compared to the group with no treatment. When increasing the concentration, stronger inhibition of the NFTs were observed, underscoring the efficacy of the COS therapy. As an added benefit, the researchers were also able to show the ability of the COS to detangle the amyloid β NFT depending on the dosage administered [37]. To investigate whether the COS could rescue neuronal functions and limit amyloid β cytotoxicity, Zhu et al. conducted immunofluorescent staining and assays to gauge apoptosis and cell viability. COS was found to be non-toxic, and treatment with COS was found to decrease cytotoxicity from 60.1% viability in control group cells (with amyloid β NFTs) to 90.1% viability in COS treated cells. Lastly, the role of COS as an anti-inflammatory agent was examined. This included the use of BV-2 cells, also used by Zhong et al., to establish the impact of COS on amyloid β caused oxidative stress and ROS production. Fluorescence assays showed a substantially lower illumination in the COS group, while the group with amyloid β showed the highest fluorescence, indicating that COS is able to decrease and inhibit the production of ROS via COS role in amyloid β binding and downstream effects on oxidative stress [37].

The findings of Zhu et al. demonstrate safety and efficacy of chitosan oligosaccharides when used for the purpose of binding amyloid β plaques and NFTs to prevent downstream inflammation and aggregation. The researchers used statistically significant results to underscore their conclusions and demonstrated possible avenues for future research. One area which was not explored but may have been able to benefit from further research in this study was the conduct of behavioral tests to confirm overall cognitive benefits. However, this research provides a solid foundation on which further research with chitosan can be built upon and hopefully continued.

Behavioral Tests in Mice Corresponding to Nanoparticle Efficacy

Most of the studies of nanoparticle efficacy for BBB delivery are done in AD mouse models, where it can be difficult to quantify the effectiveness unless explicitly measured. Typically, these tests are behavioral and are used to gauge how well the potential treatments work. The Morris Water Maze (MWM) is a test gauging the acquisition and maintenance of spatial memory. In this test, mice are introduced to a pool of water and made to find a platform. After a single day, the mice are exposed again to the same pool and location of the platform, but the platform is invisible. This is done for six days to ensure that the mice are able to acquire a spatial coordinate for the platform. This is quantified by measuring the seconds for each mouse to reach the platform for each day [38].

The novel object recognition (NOR) test is used in murine studies to test their ability to recognize new objects in their environment. There are three phases, habituation, familiarization and testing. In the first phase of habituation, the mouse is removed from its enclosure and placed in a holding tank after which it enters the phase of familiarization. In this step, the mouse is placed in an open field with two identical objects. In the test phase, the mouse is returned to the open field with a new object and one object the same as in the previous step. A mouse with an unimpaired memory would likely spend more time with the novel object in the test phase, however an impaired mouse would likely not be able to recognize that any of the objects were repeated [40]. The NOR test is similar to the open field test, where an enclosure with walls acts as an open field. This would encourage movement of the subject to wander around the enclosure. The parameters in both of these tests are distances moved, time spent moving, and change in movement patterns [41].

The 5-Choice Serial Reaction Time (5-CSRT) test is used to gauge the attention span of rats. Originally developed for human use, it has been adapted for murine studies, requiring some changes for nonverbal rule learning. This test relies on positive reinforcement for the mice to correctly identify the appearance of a light stimulus in a set number of locations [42]. The Paired Associate Learning test in mice involves learning and memory, using object placement in spatial environments. The rodents must identify the correct object in the correct position in order to demonstrate proper memory function [43].

Future Trends

There is reason to be cautiously optimistic about the future of nano-therapies for the use of treating Alzheimer's Disease. The research

discussed in this paper shows that, although preliminary, there are tangible results to build upon. However, there are substantial reasons for caution when discussing the possibilities. Nanoscale drug delivery nano-therapeutics experience difficulties in reaching the clinical space from preclinical space. It is hypothesized that much of it may be due to manufacturing defects, shelf-life stability, or physicochemical characteristics that affect the pharmacokinetics [44].

One of the largest drawbacks is that much of the research discussed in this paper is conducted *in-vitro* or in *in-vivo* murine models. Although mammalian, murine studies provide small models, and the results don't fully convey the ranges of adverse effects, safety and efficacy that is needed to translate to clinical application and approval. Additionally, it is difficult to fully recreate the pathophysiology of AD in murine studies. Much of the research discussed in this paper used APP/PS1 mice, but this is not fully representative of the spectrum of AD causes and symptoms [45]. These limited models provide a tenable foundation, but are unable to fully capture the complexities of the neurodegeneration of AD. Diehl et al. [46] describe the possibility of using bearded capuchin monkeys as a model for Alzheimer's Disease, after neuro-histological analysis showed naturally occurring amyloid β plaque aggregation and AD-pathology. Using primates could help to be a pre-clinical bridge between studies conducted with mice and first in human studies conducted in the clinic. The research outlined in this paper focuses mostly on intravenous caudal administration, however another avenue of research with immense potential is nanoparticle administration via an intranasal route. There has been previous success in this area due to the direct line along the olfactory pathway, from the nasal cavity to the forebrain. Huang et al. [47] discuss this pathway as an optimal avenue, which provides a sound way to bypass the blood barrier for the purpose of direct treatment into the brain. Dighe et al. [48] also outline previous research that has had success using polymeric nanoparticles such as chitosan, PLGA or metallic nanoparticles such as gold nanoparticles or iron oxide nanoparticles. These options would provide a route of administration that is less invasive but potent. Additionally, administration would translate well from the pre-clinical space to the clinical space where the route could remain the same.

Conclusion

Treatment for Alzheimer's Disease is remarkably sparse, treatment options must fit into specific criteria otherwise they risk being mitigated by the blood brain barrier. Nanotechnology provides an excellent alternative for traversing the blood brain barrier to treat Alzheimer's Disease. A bulk of the research is used for nanoscale drug delivery systems. Black phosphorus nanosheets have dual use as an anti-inflammatory agent and a drug delivery vehicle for drug molecules. These drug molecules are often filtered out by the blood brain barrier due to size, shape, surface charge, or chemical interactions. Black phosphorus nanosheets possess the ability to tackle oxidative stress caused by amyloid β plaque aggregation and hinder its ability to cause downstream tau hyperphosphorylation. Their function as a nano-carrier also enables them to carry more effective therapeutics that would otherwise be eliminated by the blood brain barrier. Efficacy was shown for both functions of the black phosphorus nanosheets in mice models, with histological examination of brain tissue for amyloid β aggregates, as well as confirm with behavioral tests. siRNA has been effective for its use in gene knockout therapies, however, it is easily degraded in-vivo due to fragile physicochemical characteristics, as well as the abundance of RNases in-vivo that target it for degradation. The use of synthetic polymeric nanoparticles to encapsulate siRNA for gene knockout was shown to be effective in murine models and confirmed histologically as well as with behavioral tests. Targeting hyperactive microglia with Prussian Blue encapsulated PAMAM dendrimers was also shown to be an effective target for Alzheimer's Disease. By reducing preliminary neuro-inflammation, researchers were able to turn off the positive feedback loop of hyperactivation of microglia and restore the function of microglia that had been triggered. Chitosan nanoparticles also provided an avenue for the treatment of Alzheimer's Disease due to its innate function as an anti-inflammatory. Researchers were able to confirm decreases in amyloid β aggregation and subsequent oxidative stress caused by reactive oxygen scavenging with chitosan's ability to bind to amyloid β plaques. This was confirmed with neuro-histological analysis.

The volume of research discussed in this paper provides a solid foundation on which to treat Alzheimer's Disease. Although most of the research is still taking place in a pre-clinical setting, the future for nanotherapeutics for the treatment of Alzheimer's Disease is bright. Future success is dependent on the translation to the clinical space and more specific applications to overcome the transition from murine to human studies. With this transition, more information will yield better research avenues on how to successfully treat Alzheimer's Disease in humans.

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