Nanoparticle Based siRNA Delivery to the Central Nervous System:
A Potential Therapeutic Approach

by

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Abstract

Efficient delivery of siRNA via nanoparticles may emerge as a viable therapeutic approach to treat central nervous system disorders including Alzheimer’s Disease (AD), a progressive neurodegenerative disease currently lacking effective treatment. For efficient distribution and cellular uptake of therapeutic nanoparticles, the shape of these delivery vehicles is thought to be a critical determinant. While significant progress has been made to control the morphology of nanoparticles loaded with traditional small molecule drugs, strategies to control the shape of nanoparticles encapsulating siRNAs remain a challenge. Here, we show that a linear polyethyleneimine (LPEI)-g-polyethylene glycol (PEG) copolymer-based micellar nanoparticle system to deliver siRNA, with different grafting degrees of PEG, leads to the formation of micellar nanoparticles with distinct morphologies, including worm-like, rod-like or spherical nanoparticles. In cultured (N2a) cells, we observe robust and selective knockdown of two therapeutic targets of Alzheimer’s disease (namely, BACE1 and APP) using the LPEI polymer complexed with siRNA against either BACE1 or APP. Importantly, by infusing these various shaped nanoparticles into the lateral ventricles of living mice, we show that rod-shaped nanoparticles achieved the most robust knockdown of BACE1 in their brain. Furthermore, by utilizing the circulation of the cerebrospinal fluid we show that such knockdowns can be achieved in spinal cords of these mice as well. These findings collectively indicate that the shape of nanoparticles, encapsulating siRNA, is an important determinant for their ability to efficiently affect gene knockdown and deliver their payload in the central nervous system.

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Chapter 1 Introduction
Alzheimer’s Disease

Alzheimer’s Disease (AD) is a progressive neurodegenerative disorder, first identified by the German psychiatrist - Alois Alzheimer, affecting close to 30 million people worldwide and anticipated to increase as the years go by (Holtzman, Morris et al. 2011). The National Institute of Aging (NIA) defines AD as an irreversible progressive brain disease that slowly destroys thinking and memory skills of affected individuals. AD is the most common cause of dementia and as the disease progresses it destroys the ability of afflicted individuals to carry out the most basic/simple tasks for daily living (Barker, Luis et al. 2002). Disease onset typically commences at ~60-65 years of age, at which time the patients might be diagnosed with mild cognitive impairment, followed by progressive and rapid decline in cognitive abilities (late stage AD) (Bateman, Xiong et al. 2012). Due to the very fabric of one’s being getting affected, without them being aware, the care of patient falls on the families and caregivers (who get most affected emotionally due to disease affecting their loved ones). In 2013 it was estimated that $220 billion in terms of hours of unpaid care was provided by approximately 15.5 million caregivers, with AD being the sixth leading cause of death in the United States (US) (www.alz.org). It is estimated that by 2050, 16 million cases of AD would be prevalent in the US alone (www.alz.org).

At the present time, diagnosis of the disease presents a significant challenge. Patients first need to be screened by doctors to exclude other causes for cognitive decline such as a brain tumor, thyroid disease or chronic infections (McKhann, Drachman et al. 1984). The Clinical Dementia Rating (CDR) scale has evolved as a useful metric to be able to assess the severity of dementia in patients – where a score of zero, indicates normal cognitive activity, and a score of 3 indicates significant cognitive decline (Morris, Ernesto et al. 1997). In this context it becomes essential to be able to develop a history of cognitive capability on an individual basis so as to monitor dementia onset and progression over time. Additional cognitive tests such as a mini-mental state examinations (MMSE) have also been utilized to study progression of the disease in afflicted
patients (McGrory, Doherty et al. 2014). Coupled with these examinations, biomarker studies and pathology are used to confirm an AD diagnosis.

Pathology of AD

Brains of patients afflicted with AD show gross atrophy (Ridha, Barnes et al. 2006). Hallmarks of the disease include the presence of amyloid plaques, neurofibrillary tangles (NFT) and significant neuronal loss (Choi, Kim et al. 2014). Amyloid plaques comprise the Aβ peptides deposited in the extracellular space of neurons. Aβ peptides are derived from the processing of the amyloid precursor protein (APP) (Hardy and Selkoe 2002). The amyloid plaques deposited in the extracellular space can be composed of Aβ present as fibrillar and oligomeric species (Kayed, Head et al. 2003). These amyloid plaques are surrounded by degenerating axons and dendrites also depict activation of astrocytes and microglia and Aβ deposits have also been found in blood vessels and the condition is known as cerebral amyloid angiopathy (von Bernhardi and Ramirez 2001, Smith and Greenberg 2009).

In contrast to Aβ plaques that are found in the extracellular space of neurons, NFTs found in neuronal cell bodies and processes are also representative of AD pathology. The intracellular NFTs are made up of a hyperphosphorylated form of tau- a microtubule-binding protein (Wang, Xia et al. 2013). The normal function of tau includes binding tubulin and stabilizing microtubules, in neuronal and glial cells, that are essential for transport of cargo in an anterograde and retrograde manner (Zhang, Carroll et al. 2012). While normal tau is very soluble, hyperphosphorylated tau dissociates form microtubules and has a propensity to aggregate, leading to the formation of NFTs. These aggregates appear as paired helical filaments composed of proteins in a β-sheet structural conformation (Goedert, Spillantini et al. 1992). A significant body of evidence suggests that the Aβ and NFT pathology are significant players contributing towards neuronal dysfunction and progression of AD (Bateman, Xiong et al. 2012). Each of these proteins is toxic to the cells present in the central nervous system (CNS). Neuronal populations that have
been identified to be particularly vulnerable to in AD include those present in the hippocampus and frontal, temporal and parietal cortex (Du, Schuff et al. 2007). In this context it is interesting that studies have shown the spreading of Aβ and NFTs, in the CNS, in mouse models which suggests that these proteins are capable of spreading in a prion protein like manner (Clavaguera, Bolmont et al. 2009, Frost and Diamond 2010). While Aβ species are present in the extracellular space of neurons and normal secreted from synapses, the spread of NFTs is very interesting. Although NFTs have been identified as predominantly intracellular proteins, their spread across broad regions of the brain has been observed using microdialysis to sample the interstitial fluid in the brain, thus throwing light on possible mechanisms by which tau species might migrate in the CNS (Yamada, Cirrito et al. 2011). Tau aggregation plays an essential role towards progression of AD. Tau aggregation can lead to neurodegeneration independent of Aβ accumulation in the brain. But, in the context of AD, evidence suggests that part of tau related neurodegeneration can be attributed to early Aβ deposition in the brain (Bateman, Xiong et al. 2012).

Aβ deposition in the form of amyloid plaques is known to develop for years prior to initial manifestation of cognitive deficits. Followed by the increasing presence of NFTs the double hit is thought to rapidly lead to neurodegeneration followed by neuronal loss. Indeed in patients, studied in Iceland, a “protective” mutation was discovered that reduced the processing of APP into Aβ species that delayed onset of the disease further strengthening the argument for the amyloid cascade hypothesis that suggests that Aβ species is the key player leading to development of AD followed by NFTs that have been shown to be independently toxic in other disorders such as Frontotemporal Dementia and other tauopathies (Jonsson, Atwal et al. 2012).

**Genetics of AD**

In a small percentage of families it has been observed that AD can be inherited. In particular, mutations in the APP gene have been implicated in terms of affecting the cleavage by the β-secretase and γ-secretase and additional mutations have been found in the presenilin-1 (PS1),
component of the \( \gamma \)-secretase complex, have been linked with increase in the total \( \text{A}\beta_{42} \) levels as well (Holtzman, Morris et al. 2011). Importantly, the presence of the APOE\( \varepsilon4 \) allele has been clearly implicated as a genetic risk factor where the risk of being affected by AD goes by by a factor of 12 fold for those with two copies and 3 fold for those with one copy (Corder, Saunders et al. 1993). These familial AD cases provide a rich body of evidence for the key role that \( \text{A}\beta \) plays in the amyloid cascade hypothesis.

**APP Processing and Strategies for Therapeutic Development**

APP is an integral membrane protein with high expression levels in the CNS (Theuns and Van Broeckhoven 2000). The \( \text{A}\beta \) protein that aggregates in the brain of AD patients is derived from the successive cleavages of APP by \( \beta \)-secretase and \( \gamma \)-secretase (Chang, Huang et al. 2011). These cleavages lead to the release of the APP intracellular domain (AICD) and \( \text{A}\beta \) product that is secreted from the cells as a part of their normal function. Alternatively APP can also be cleaved by the \( \alpha \)-secretase and \( \gamma \)-secretase. The processing of APP by \( \alpha \)-secretase is at a site downstream from the \( \beta \)-secretase cleaving sites and leads to the formation of smaller products such as the \( \text{A}\beta_{17-42} \), \( \text{A}\beta_{17-40} \), and P3 (where the amino acid cleaved by the \( \beta \)-secretase is labeled as ‘1’ – start of the numbering sequence) (Holtzman, Morris et al. 2011). Cleavage of APP in this manner forms a part of the non-amyloidogenic pathway that does not lead to harmful species that form \( \text{A}\beta \) deposits in the brain. Cleavages by the \( \beta \)-secretase and the \( \gamma \)-secretase lead to the formation of a variety of species such as \( \text{A}\beta_{38} \), \( \text{A}\beta_{39} \), \( \text{A}\beta_{40} \), \( \text{A}\beta_{42} \), and \( \text{A}\beta_{43} \) (Wolfe 2012). Since the \( \beta \)-secretase cleaves at a very specific site, the previously mentioned \( \text{A}\beta \) species are formed by the cleavage at different sites by the \( \gamma \)-secretase complex. Additionally \( \gamma \)-secretase has another cleaving site called ‘\( \varepsilon \)’ which is located at amino acid 49. Among these \( \text{A}\beta \) species \( \text{A}\beta_{40} \) is the one that is most abundantly found in the brain. \( \text{A}\beta_{40} \) levels can also be measure in the cerebrospinal fluid (CSF) that bathes the CNS (Seubert, Vigo-Pelfrey et al. 1992, Cirrito, May et al. 2003). The \( \text{A}\beta_{42} \) species is considered to be more prone towards forming fibrils that deposit first in the brain (Ahmed,
Davis et al. 2010). Although the total amount of Aβ42 is less than the amount of Aβ40, the increasing ratio of Aβ42/ Aβ40 over time is considered to be key metric essential for Aβ pathogenesis.

Interestingly in experiments observing the processing of APP by the γ-secretase complex it was noted that the larger forms of the peptide, such as, Aβ48 and Aβ49, were processed into shorter forms such Aβ45, Aβ42, Aβ38, and Aβ46, Aβ43, Aβ40 respectively (Wolfe 2012). This interesting cleavage of the peptide every three amino acids is purely a function of the γ-secretase complex. Considering the critical role that the enzyme plays in the generation of Aβ its inhibition has been the focus of several therapeutic efforts towards alleviating AD in humans. Initial compounds that were developed towards inhibiting γ-secretase were very effective in reducing the activity of the enzyme. In addition to affecting Aβ generation the cleavage of notch, another substrate, by γ-secretase was also compromised (De Strooper, Annaert et al. 1999, Curry, Reed et al. 2005).

Cleavage of notch is known to be critical towards determining cell fate and as such its inhibition leads to the development of toxic side effects such as tumor generation in the skin (Li, Wen et al. 2007). Importantly, while such a phenotype was also observed in preclinical studies involving these γ-secretase inhibitors tested in the mice, they progressed to human clinical trials. These trials involving the use of γ-secretase inhibitors had to be halted due to toxic side effects and more importantly it was observed that the cognitive ability of the patients receiving the drug worsened compared to that of the placebo treated cohort (Schor 2011). It is speculated that the inhibition of the critical function of notch might have been a contributor towards cognitive detriment. To overcome the challenge of selectivity between substrates, γ-secretase modulators have been developed (Kounnas, Danks et al. 2010). This class of compounds has been shown to selectively affect the generation of Aβ42 while sparing the notch function of the secretase. By decreasing the ratio of Aβ42/ Aβ40 and shifting production towards the shorted peptide species that do not aggregate and are thought to be non-toxic, has lead to the hope of these compounds having a better efficacy and safety profile in clinical trials. The exact mechanism by which selectivity is
achieved or an explanation for why exactly only one particular cleavage step is affected, in the
triamino acid processing of the peptide by γ-secretase, remains to be better understood.

**BACE1 Therapeutic Development**

The β-site APP cleaving enzyme 1 (BACE1) is transmembrane aspartic protease. The active site
is made up of two aspartic protease motifs that are oriented towards the lumen of intracellular
compartments of the cell (Yan and Vassar 2014). BACE1 is highly expressed in neurons and
requires an optimal acidic pH for enzymatic activity (Cai, Wang et al. 2001, Cole and Vassar
2007). Considering the key role that BACE1 plays towards generating the key Aβ species,
implicated in AD pathogenesis, development of its pharmaceutical inhibitors has been relentlessly
pursued. Several candidate inhibitors have entered clinical trials. At present no therapeutic drug
has been approved for AD treatment. Particular attention is being paid towards understanding
untoward mechanism based side effects due to global inhibition of BACE1.

The initial motivation for pursuing BACE1 inhibition as a therapeutic strategy was driven by the
observation that BACE1−/− mice appeared normal with no behavioral/clinical deficits (Cai, Wang
et al. 2001, Luo, Bolon et al. 2003). Upon crossing these mice with APP transgenic mice, that
develop Aβ plaques, it was observed that Aβ production was abolished coupled with overcoming
of memory deficits (Ohno, Cole et al. 2007). It was also noted, based on these reports that
BACE2, which is homologue of BACE1, did not compensate for loss of BACE1 activity. But,
more recent careful examinations of the BACE1−/− mice have revealed behavioral deficits similar
to those of schizophrenia, hypomylenation of neurons, seizures, and retinal pathology which
suggests that coupled with inhibitor development, a more fundamental understanding of BACE1
functions would be important as one pursues this therapeutic approach (Laird, Cai et al. 2005,
Savonenko, Melnikova et al. 2008).

As BACE1 emerged as a strong therapeutic target, following validation studies in *in vitro* and *in
vivo* models, initial efforts focused on inhibitor development that mimicked the peptide transition
state analogues at the BACE1 cleavage site (Hong, Turner et al. 2002). While early results were promising, the bioavailability of the drug in CNS was limited due to lack of transport across the blood brain barrier (BBB). Since AD treatment would require long term administration of the drug, it is essential for these molecules to have suitable characteristics to be able to penetrate the BBB in a safe manner.

An improved class of compounds was developed after the crystal structure of the enzyme with the inhibitor was elucidated (Hong, Koelsch et al. 2000). Rapid progress since then has been made towards characterizing these compounds in preclinical studies and they have progressed on to human clinical trials. A compound from the company Merck, MK-8931, has progressed to Phase 2/3 in clinical trials and shown to be well tolerated in patients with reductions in Aβ species (75-90% depending on the dose) (Baker 2013). This drug, by its mechanism of action, has also shown the capability of being able to reduce Aβ levels in patients in advance stages of AD with significant Aβ deposits. It is important to recognize that while clinical trials involving the above mentioned drug have been successful, other trials have failed after showing promising results in preclinical studies (Yan and Vassar 2014). Retinal pathology and liver toxicity have been the cause of a trials being stopped for drugs from other companies while other newer candidates enter Phase 1 trials. The side effects observed have been attributed to the drug itself and not related to the targeting of BACE1. In contrast to the story of drug development for γ-secretase inhibition, in which toxicity was due to target inhibition, BACE1 inhibition continues to remain an attractive therapeutic target.

While it is recognized that treatment of AD patients on drugs would be long term we need to be able to have answers to determine what level of enzymatic activity reduction would be required and at what stage in the disease progression spectrum would patients most benefit from the treatment regimen. A recent mutation (Ala673Thr) found in the Icelandic population has been found to be protective in the context that BACE1 cleaves APP less efficiently thus leading to
approximately 40% reduction in Aβ levels (Jonsson, Atwal et al. 2012). Individuals heterozygous for the mutation show 20% reduction in Aβ levels over long spans of time. From mouse models we have learnt that an approximately 50% inhibition of BACE1 leads to a 20% reduction in activity (Rabe, Reichwald et al. 2011). This data is promising for researchers in academia and industry since current candidates in trials achieve Aβ reductions well beyond the 20% level. The level of BACE1 reduction that has currently been achieved would benefit patients with almost twice the level of BACE1 expression and bring them back to levels of those of non-demented patients. But, it is important to recognize that the protective mutations in the Icelandic population had been present for years, which leads to lower Aβ loads over a substantial period of time. Longitudinal studies, involving individuals enrolled in the Dominantly Inherited Alzheimer’s Disease Network (DIAN), have shown that changes in Aβ_{42} levels in the CSF can be observed 25 years prior to symptomatic manifestations of the disease (Bateman, Xiong et al. 2012). Aβ deposits were detected in the brain, using Pittsburg compound B, 15 years prior to symptom onset coupled with increases in levels of the protein tau and brain atrophy. This data indicates a tremendous challenge towards being able to design/administer a therapeutic several years prior to any manifestations of symptoms of the disease. On the other hand it indicated that by the time patients are diagnosed, substantial damage in the brain has already occurred. Early clinical trials involved treating patients in the late stage of AD and did not show significant benefit for patients. Current trials have recognized this aspect of intervention time being a key factor towards halting AD progression and have started to include patients in the prodromal phase of the disease. With these new approaches being employed, the field is hopeful that we might better be able to judge the targeting of BACE1, a key player in the amyloid cascade hypothesis.

Aβ immunotherapy has also emerged as a potential therapeutic approach for AD. It has been shown to enhance clearance of soluble and aggregated Aβ and improve learning and memory in preclinical studies (Janus, Pearson et al. 2000). A trial involving testing the Aβ immunization
approach in humans had to be halted after a few patients developed meningoencephalitis (Check 2002). Passive immunotherapy against Aβ continues to be pursued and recruiting appropriate patient populations in this context will once again be critical. Also essential would be to understand the exact mechanism of action of the immunotherapy approach. While the “sink hypothesis” has been proposed to explain its mechanism of action, convincing evidence in this regard continues to be investigated (Lobello, Ryan et al. 2012). Next generation antibodies have also been engineered to fuse with antibodies targeting the transferrin receptor, which have been tuned to improve BBB transcytosis and thus improve brain distribution (Atwal, Chen et al. 2011). While the quest for a suitable therapeutic continues there continues to remain a significant unmet need in terms of available therapeutics for challenging disorders of the CNS such as AD.

**RNAi Therapy**

The phenomenon of RNA interference was observed while studying the coloring pattern in the petunia, where the gene encoding chalcone synthase was being manipulated (Napoli, Lemieux et al. 1990, Matzke and Matzke 2004). While trying to produce a more intense color in the flowers by introducing additional copies of the chalcone synthase gene, it was observed that there was a loss of color and the formation of a banding pattern. This indicated that through some unknown process, at that time, the activity of both endogenous and exogenous genes were substantially reduced. Similar results began to be observed in the field of plant virology where the incorporation of short viral RNA sequences showed resistance to viral infections (Covey and Al-Kaff 2000). At which point the phenomenon was labeled as post transcriptional gene silencing. In 1998 Craig C. Mello and Andrew Fire published a report explaining that the gene silencing effect, observable in *C. elegans*, was on account of double stranded RNA (Fire, Xu et al. 1998). They coined the term RNA interference (RNAi) to explain the phenomenon.
By designing appropriate double stranded RNA sequences it was shown that one might target a variety of endogenous proteins in mammalian cells. The potential for this method to develop as a therapeutic approach against a host of disorders in which aberrant proteins are expressed was quickly recognized. Several research endeavors commenced towards developing RNAi as a therapeutic approach. But, it was quickly realized that greatest challenge towards this therapeutic maturing was the ability to safely package and deliver the RNA payload in a targeted manner to organs/cells of interest (Castanotto and Rossi 2009). Short interfering RNA (siRNA), ~21-25 nucleotides long, that has not been chemically stabilized, has a short half-life in the bloodstream, is unable to gain entry into cells and can trigger an immune response (Whitehead, Langer et al. 2009). siRNA must gain access to the cytoplasm of cells in order for them to be able to behave as effective gene silencers. In the cell cytoplasm siRNA is loaded into the RNA-induced silencing complex (RISC) (Schwarz, Huttvagner et al. 2003). The two RNA strands are separated, where by the guide strand (effector of gene silencing) remains loaded in the RISC and the passenger strand is degraded. The guide strand, while loaded in the RISC, is capable of binding with complementary mRNA sequences, which then leads to the degradation of mRNA (and can thus act as a means of reducing levels of the encoded protein). Via this mechanism of action one can now design a variety of siRNA sequences targeting mRNA sequences encoding protein targets of interest.

**Strategies Developed to Improve siRNA Delivery**

An ideal system for delivery of siRNA would have the following attributes: protection from degradation by serum nucleases, minimal interactions with proteins in the circulation, lack of immune response activation, extended half life of circulation - for example reduced renal clearance, the ability to be transported from the bulk circulation to the specific site of interest with lack of interaction with the non-targeted cells (Kanasty, Dorkin et al. 2013). Significant effort has been directed towards improving the stability of siRNA in *in vivo* conditions. Chemical
modifications such as the 2’-O-methyl and 2’-fluoro on the siRNA rendered them to be significantly more stable than their unmodified counterparts (Kenski, Butora et al. 2012). Viral vector platforms have been extensively studied so as to take advantage of their evolutionary capacity to deliver genetic material. Significant testament to progress in the field are the current ongoing clinical trials using viral vectors for several hard to treat disorders such as amyotrophic lateral sclerosis (ALS) using the adeno associated virus (AAV) platform (Patel, Kriz et al. 2014). But, risks such as insertional mutagenesis and immune response continue to persist, which creates a need for the continued pursuit for developing of an attractive non-viral delivery strategy (Gray, Nagabhushan Kalburgi et al. 2013). Nanoparticle delivery systems involving the use of polymers and lipids have been extensively studied as a means of overcoming the inherent challenges of in vivo delivery (Wolfrum, Shi et al. 2007, Ballarin-Gonzalez, Ebbesen et al. 2014). The positive charge on the polymer backbone is capable of interacting with the negative charge of the siRNA and form complexes. In this context it is important to recognize that the surface charge of the complex can play an important role towards determining its delivery efficiency. Nanoparticles displaying surfaces with a significant positive charge adsorb negatively serum proteins and can trigger an immune response or lead to the form of aggregated complexes in circulation. Opsonization by the mononuclear phagocytic system leads to the clearance of nanoparticles from circulation rendering them ineffective (Alexis, Pridgen et al. 2008). One of the techniques developed to overcome these issues involves decorating the surface nanoparticles with polyethylene glycol (PEG) (Hatakeyama, Akita et al. 2011). The use of this hydrophilic polymer reduces the adsorption of serum proteins on to the surface of nanoparticles. This in effect imparts ‘stealth’ properties to the nanoparticles that can now circulate for an extended period of time without being cleared as compared to their non-PEGylated counterparts. Additionally, delivery systems for siRNA must also have attributes that minimize their renal clearance, since it is known that naked siRNA can be cleared from the body efficiently by the glomerular filtration system of the kidneys (Huang, Hong et al. 2011). While naked siRNA can be efficiently filtered out from
the circulation, nanoparticles/delivery vehicles that are larger in size should not, in theory, be easily filtered in the glomerular capillaries. Disruption of nanoparticles formed by the interaction between polymers and siRNA, has also been observed, which leads to free siRNA release (Zuckerman, Choi et al. 2012).

Thus far the nanoparticle delivery strategy has been particularly successful in delivering payload to liver and cancerous tumors (Wang, Langer et al. 2012, Yu, Hsu et al. 2012). The liver has evolved as a prime target for siRNA therapeutics since it is a fundamental organ through which filtration of the blood occurs. Nanoparticles that pass through the fenestrations of the liver are retained and thus gain access to a variety of cell types in the liver. In the case of tumors it was observed that they developed newer blood vessels that are leaky coupled with inadequate lymphatic drainage. This phenomenon – enhanced permeation and retention (EPR) effect, has been duly exploited as a means of selectively targeting tumors that provide for a lot more nanoparticles to be transported from the endothelium to the tumor cells and be retained at their site of action (Wang, Langer et al. 2012).

The tremendous progress that has been made towards realizing the potential for RNAi therapeutics, and accompanying delivery strategies, is reflected in the number of human trials that are currently ongoing using this strategy (Kanasty, Dorkin et al. 2013, Tabernero, Shapiro et al. 2013). In fact the first demonstration of RNAi in humans was shown in the case of patients being treated for cancer being injected with cyclodextrin polymer nanoparticles carrying siRNA as a payload targeting ribonucleotide reductase subunit 2 (RRM2) (Davis, Zuckerman et al. 2010). It must be recognized that while initial formulations worked well in in vitro targeting studies optimization strategies were required prior to in vivo use in preclinical studies (a theme we will revisit later). The use of the previous mentioned PEGylation strategy was deployed to improve the half-life of the particles and a targeting ligand such as transferrin was used to improve targeting specificity. Over the past decade the development of lipid nanoparticles for the transport/delivery of siRNA has evolved as an effective therapeutic strategy (particularly
employed by Alnylam and Tekmira Pharmaceuticals in clinical trials). A wide array of disorders such as transthyretin-mediated amyloidosis, hypercholesterolaemia and cancer have been targeted using this delivery approach (Phase 1 & 2 of clinical trials) (Kanasty, Dorkin et al. 2013). Lipid mediated delivery of siRNA have to lead effects such as activation of the complement cascade (Moghimi and Hamad 2008). Minimization of this effect using PEG has led to continued development of this therapeutic approach. Interestingly, a deeper understanding of the structure function relationship of siRNA has lead to development of naked siRNA sequences which have been sufficiently stabilized and have a targeting ligand. These molecules have passed the preclinical development phase and entered human clinical trials. For example the use of N-acetylgalactoseamine (GalNac) technology by Alnylam Pharmaceuticals for their liver targeted therapy to target transthyretin-mediated amyloidosis (Nair, Willoughby et al. 2014). Using a similar approach of using optimizing naked oligonucleotide sequences, antisense technology (prominently developed by ISIS pharmaceuticals) has evolved as a mature platform for mediating RNAi (Yu, Pendergraft et al. 2012). Unlike siRNA, whose knockdown effect is mediated by the RISC in the cytoplasm, antisense oligonucleotides use the RNAase pathway that leads to the degradation of the transcript when a complementary DNA-RNA hybrid species is formed. The advancement in antisense technology as a viable therapeutic is reflected in the two drugs that have received approval for human use (to treat hypercholesterolemia and inflammation) and several others in the pipeline targeting cardiovascular, metabolic, cancer and inflammation targets. The diversity of the targets (and diseases) being targeted by using this approach is tremendous testament to the initial promise of using RNAi therapy as a means of targeting a wide variety of targets that have thus remained elusive to current drug development strategies. It is interesting to note that although these highly optimized sequences have shown efficacy in their naked form (with suitable chemical modifications) one can anticipate an appreciable increase in their efficacy when they are delivered using a nanoparticle platform.
Nanoparticle Approach Developed on a Polymer Platform

Nanoparticles have been extensively studied as a vehicle towards being capable of packaging and delivering DNA as a payload (Gary, Puri et al. 2007). The extension of these results in the context of siRNA delivery remains challenging. siRNA that is being delivered for therapeutic purposes is much shorter compared to DNA (21-25 base pairs as opposed to kb range for DNA). Additionally RNA adopts the A-form conformation as compared to DNA traditionally in the B-form, which results in a greater twist and compaction of its structure (Zheng, Pavan et al. 2012).

Polyethyleneimine (PEI) has been one of the most broadly studied polymer type in the context of being able to package and deliver siRNA (Dominska and Dykxhoorn 2010). While the use of branched PEI (bPEI) has been reported to show gene knockdown effects, it was shown that in an in vivo context there was a significant immune response triggered on account of these nanoparticles (Zintchenko, Philipp et al. 2008). In this context the Linear PEI (LPEI) was developed to overcome the challenges involved in the use of BPEI (Shim and Kwon 2009). The use of this formulation demonstrated the ability to overcome the safety concerns, associated with BPEI, but was observed to form severe aggregations under in vivo conditions. Once again the use of PEG to decorate the nanoparticle surface significantly reduced the aggregation tendency of these nanoparticles. Additional polymers have also extensively been studied for efficient packaging and delivery of siRNA. For example, poly(β-amino) esters have also been demonstrated to have high transfection efficiency, low toxicity, and biodegradability in the context of knocking down their targets by ~90% in a glioblastoma cell line (Tzeng, Guerrero-Cazares et al. 2011). Gene knockdown has also been observed using a PEI-graft-poly(caprolactone)-block-PEG (PEI-g-PCL-b-PEG) copolymer where the PCL serves as the biodegradable component (Zheng, Librizzi et al. 2012). Nanoparticles formed with this formulation lead to a knockdown of ~75% in SKOV3, a human ovarian cancer cell line, as opposed to ~50% reduction with the use of PEI alone. Building on this strategy of incorporating a hydrophobic component in the polymer backbone, poly(D,L-lactic acid-co-glycolic acid) (PLGA)
was used to stabilize nanoparticles. This system leads to the formation of nanoparticles in the 30 nm range and efficiently reduce gene expression by ~60\% \textit{in vitro} (Lee, Mok et al. 2011). An interesting study detailed the use of iron oxide nanoparticles coated with molecules similar to lipids so that they may be able to efficiently interact with DNA and siRNA. It was shown that by using iron oxide particles, they could be tracked using magnetic resonance imaging. Importantly by using magnetic field it was shown that these nanoparticles were capable of knockdown their targets by ~90\%, in a cell culture model, as opposed to ~50\% knockdown in the absence of a magnetic field (Jiang, Eltoukhy et al. 2013). This opens up avenues to continue exploring the efficient knockdown using external stimuli to guide and mediate knockdown in a variety of complex systems. Side chains of amino acids that are ionizable, to have a positive charge, have also been used to electrostatically interact with negatively charged siRNA (for complexation purposes). A PEG-\textit{block}-poly(L-lysine) copolymer based nanoparticle system, that was stabilized with disulfide crosslinking, by modifying the side chains with Trauts reagent, was shown to significantly increase the stability of nanoparticles in serum containing media as compared to non-stabilized particles (Matsumoto, Christie et al. 2009). The disulfide crosslinking strategy has been widely used to maintain the integrity of nanoparticles in the oxidizing environment of the circulation and conveniently release its payload under reducing conditions of the cell. Additional studies have highlighted the use of this strategy to prepare siRNA loaded in nanoparticles based on PBAEs, which displayed enhanced stability and knockdown capabilities as compared to lipid reagents (Lee, Green et al. 2009). Gold nanoparticles were first modified with PEG, and siRNA was conjugated via a disulfide crosslinker, which was then coated on the surface with PBAEs. This multistep process of particle formation displays the versatility of using this stabilization method for the key siRNA payload. An ionic crosslinking reagent such as tripolyphosphate (TPP) in conjunction with the PEG-\textit{block}-polyphosphoramidate was shown to be able to efficiently package siRNA, form particles that were ~100nm and able to achieve efficient gene knockdown
in HeLa and D407 cells (Nakanishi, Patil et al. 2011). In this particular system, in the absence of TPP no coherent particle formation was detected.

While the packaging and stabilization of nanoparticles represent critical initial steps towards realizing the potential for RNAi therapy, significant effort has been put forth to understand the mechanisms of cell uptake of nanoparticles coupled with strategies towards improving the efficiency of this step. The familiar strategy of PEGylation mentioned above has also been used in this case to mask the positively charged species formed at the end of the complexation process (which have a proclivity to aggregate in biological media). While PEG is successful at mitigating the aggregation of nanoparticle species the efficiency of cell uptake is reduced (Mishra, Webster et al. 2004). Significant efforts have been focused towards improving cell uptake utilizing strategies to make particles that would be responsive to stimuli in the tumor microenvironment, such as the drop in pH to slightly acidic conditions and abundant presence of matrix metalloproteinase (MMP) (Hatakeyama, Akita et al. 2011). Utilizing crosslinkers between the polymer backbone and PEG that are pH sensitive and cleavable by MMPs, it was shown that these particles, that were responsive to external stimuli, showed enhanced cell uptake and gene knockdown.

Targeting ligands have been extensively studied as a means of improving cell uptake and targeted delivery of payload to various organs/tissues. RGD, transferrin, folate, GalNac, cell penetrating peptides (CPPs), HIV derived TAT peptide, rabies virus glycoprotein (RVG), anti-transferrin receptor antibody, anti insulin receptor antibodies are a few examples of targeting ligands that have been developed and extensively studied. These targeting ligands have shown increased cell uptake of nanoparticles coupled with improved gene knockdown capabilities (Cho, Wang et al. 2008). While these targeting ligands have been individually studied in the context of improving targeting capability, multifunctional particles have also been developed so as to improve cell targeting and uptake (Sanvicens and Marco 2008). The use of the RVG and anti-transferrin/insulin receptor antibody technology has been shown to allow for the transcytosis of
cargo across the blood brain barrier (Pardridge 2008, Alvarez-Erviti, Seow et al. 2011). The successful development of ligand targeted delivery holds the potential for allowing RNAi therapeutics to realize its immense potential of targeting a broad set of disorders that currently lack treatment, for example - neurodegenerative disorders such as AD, Huntington’s Disease. While much progress has been made towards understanding a variety of parameters involved in nanoparticle design, a continued effort of tuning nanoparticle characteristics would be essential towards the development of optimal siRNA delivery carriers for targeting various organ systems in vivo.

**Shape Controlled Nanoparticles**

As stated above significant progress has been towards improving the efficiency of packaging siRNA and release of payload using the nanoparticle strategy. Towards improving the efficiency of this delivery strategy, shape of the nanoparticle carriers is one of the key parameters that is being studied extensively and appreciated in the context of in vitro and in vivo delivery. Lithography based techniques have been applied to particle formulations based on poly(lactic acid) and poly(lactic acid-co-glycolic acid) to form worm-like, cubic and cylindrical shapes with a variety of aspect ratios (Enlow, Luft et al. 2011). These nanoparticles have been shown to be capable of packaging a variety of therapeutic agents. While shape control on a laboratory scale might be successful, broader application of these nanoparticles in clinical setting would require the use of techniques that would be amenable towards scaled up production. Membrane stretching methods have also been successfully applied towards transforming the shape of spherically shaped polystyrene into rods and elliptically shaped disks (Champion, Katare et al. 2007). But, the multiple steps involved such as liquefying, heating, and then stretching would need to be optimized for purposes of scaling up as well. By conjugating antibodies, targeting ICAM-1 and the transferrin receptor, these differently shaped particles showed the ability to accumulate in the lung and brain vasculature preferentially (Kolhar, Anselmo et al. 2013). It is in this aspect that
one can truly harness the ability of distinctly shaped particles towards specifically targeting a variety of tissue types. Since the surface area of contact would change between the ligands present on a spherically shaped particle as opposed to a rod-like or worm-like particle, one can imagine that the specific local interactions of these particles would play an important role towards improving targeting and delivery attributes. Additionally, it has also been observed, in the context of epithelial and endothelial cells, that nanodiscs showed greater internalization as compared to nanorods (Agarwal, Singh et al. 2013). These observations further emphasize the point that different shapes can play an important role in the context of targeting a variety of organ systems.

Using solvent polarity as a strategy to control nanoparticle shape, with a PEG-b-polyphosphoramide(PPA) polymer, it was shown that the morphology of these shaped nanoparticles could be exquisitely tuned to form spherical, rod-like and worm-like species (Jiang, Qu et al. 2013). Importantly, in the context of delivery of DNA to the liver it was shown that the worm shaped nanoparticles were most efficient as compared to the other two morphologies.

Using poly(caprolactone) to deliver paclitaxel in rodent models, it was observed that worm shaped ‘filomicelles’ were able to circulate for a up to a week which was substantially longer than what was observed using 200 nm shaped spheres (Geng, Dalhaimer et al. 2007). The researchers highlight the fact that longer worm like particles were successful at being able to evade clearance by macrophages when present in the circulation since they mimicked the size scale of a red blood cell.

Interestingly DNA strands have been used as a building block to generate a variety of complex two- and three-dimensional structures that have also been used for therapeutic delivery applications, including small molecule drugs, antibodies, nucleic acids and have also been shown to be less resistant to degradation (Hahn, Wickham et al. 2014). A DNA tetrahedron was constructed via complementary base-pairing of single stranded oligonucleotides, and was shown to be able to package and deliver siRNA to silence genes of interest in a tumor model (Lee, Lytton-Jean et al. 2012). An important advantage this method afforded is allowing one to
precisely control the density and the spatial orientation of ligand placement with the goal of being able to tune these parameters to improve transfection efficiency. Results have been extended from these pioneering studies to show that RNA can also be used as a building clock to assemble a variety of higher order structures. Along the lines of earlier efforts an *in silico* design strategy was employed to create nanocubes and nanorings that were able to continue to function as RNA nanoparticles (Afonin, Kasprzak et al. 2014). Factors that affect naked RNA delivery *in vivo*, such as lack of cell uptake, rapid clearance and degradation by nucleases also play a role in reducing the efficiency of these higher order RNA structures that were capable of packaging multiple siRNAs. In order to address these concerns cationic amphiphiles, that have been widely used, were employed as vehicles to effectively deliver RNA. When complexed with an amphiphile the uniquely shaped RNA structures were condensed into spherically shaped micelles. In order to overcome the challenges involved with RNA stability, oligomerized RNA sequences have been generated, from a circular DNA template, to form RNA microsponges (~500 nm), made up of densely loaded RNA ( Shopsowitz, Roh et al. 2014). RNAi microsponges successfully complexed with lipofectamine and LPEI to form condensed nanoparticles which were successful in mediating gene knockdown in an *in vitro* and *in vivo* mouse tumor model. While shape control using DNA and RNA as building blocks has been possible, controlling the shapes of nanoparticles following condensation of these species with polycations remains a challenge that needs to be addressed.
Our Rationale for Shape Controlled siRNA Nanoparticle Approach to Target AD

Genetic studies have shown that moderate reductions in BACE1 and γ-secretase have a significant effect towards affecting amyloidosis (Chow, Savonenko et al. 2010). The pharmaceutical industry has invested significantly in the development of BACE1 and γ-secretase inhibitors towards attenuating amyloidosis. But, due to mechanism based toxicities and off-targeting effects in clinical trials the programs had to be halted (Imbimbo and Giardina 2011, Cai, Qi et al. 2012). Thus, there is an urgent need to develop potential methods to modulate these targets of interest as we are faced with a burgeoning aging population.

As mentioned before the RNA interference (RNAi) therapeutics has matured from the initial studies in plants and then *C.elegans* to studies involving clinical trials with patients (Kanasty, Dorkin et al. 2013). For the promise of RNA therapeutics to be realized significant progress has been made to package short interfering RNA (siRNA) species of interest using cationic polymers to form nanoparticles that can protect siRNAs from rapid degradation and enable them to be delivered to target cells. As highlighted above this siRNA delivery strategy has been particularly successful in areas such as cancer, where the enhanced permeation and retention effect can be exploited as a means of selective delivery, and liver targeted delivery (Coelho, Adams et al. 2013, Lee, Yoon et al. 2013). A recent study showed the use of a gold nanoparticle platform to deliver siRNA to target the antiapototic pathway in glioblastoma multiforme (GBM) *in vivo* mouse models (Jensen, Day et al. 2013). Significant progress has been made to reduce the immunogenicity of viral carriers for the purposes of gene therapy in the CNS (Gray, Nagabhushan Kalburgi et al. 2013). We focused on delivering siRNA using a non-viral delivery strategy so as to mitigate the safety concerns associated with viral vectors. Non viral carriers have been shown to have a good safety profile and have been employed for delivery of siRNA in humans (Davis, Zuckerman et al. 2010, Lee, Yoon et al. 2013). More recently antisense therapy, and the development of single stranded optimized siRNA sequences, has emerged as an alternative method that was shown to knockdown proteins implicated in the CNS of Huntington’s
Disease (HD) and Tau proteins in mouse models (Yu, Pendergraff et al. 2012, DeVos, Goncharoff et al. 2013).

Nanoparticle shape has been shown to be key parameter towards improving the delivery efficiency (Venkataraman, Hedrick et al. 2011). Studies have shown how different shapes utilize differing mechanisms to gain entry into cells (Gratton, Ropp et al. 2008, Agarwal, Singh et al. 2013). While exciting progress has been made in terms of manipulating RNA sequences into a variety of geometries, shape control of the RNA species encapsulated within nanoparticles has been inaccessible (Afonin, Kirkeeva et al. 2012, Afonin, Kasprzak et al. 2014, Shopsowitz, Roh et al. 2014). A recent study has shown that rod shaped particles, showed better accumulation in the lung and brain vasculature when infused intravenously, compared to spherically shaped particles (Kolhar, Anselmo et al. 2013). Despite recent advances, manipulation of the shape of siRNA loaded nanoparticles, and a study of their efficacy in the context of delivery to the CNS remain to be addressed. Here we demonstrate the use of linear polyethylenimine (LPEI, \( M_n = 17 \text{ kDa} \)) to encapsulate siRNA targeting BACE1 and APP (two key players implicated in the amyloid cascade hypothesis), and knockdown these targets in an N2a cell culture model. LPEI has been demonstrated to have a better safety profile compared with branched PEI (Bonnet, Erbacher et al. 2008). Additionally, when we varied the grafting degree of polyethylene glycol (PEG, \( M_n = 10 \text{ kDa} \)) on the LPEI\(_{17k}\) backbone, and used these PEGylated polymers to complex with siRNA, we formed worm, sphere, and rod shaped nanoparticles. To ensure the \textit{in vivo} delivery of the siRNA to the cytoplasmic compartment of cells, we incorporated a widely used disulfide crosslinking strategy to stabilize the nanoparticles (Jiang, Zheng et al. 2010). We also verified the ability to tune the shapes of these particles by simply varying the parameter of the mixing ratio of the polymer and siRNA. We then evaluated the \textit{in vivo} efficacy and safety of these shaped nanoparticles in terms on targeting BACE1 in the CNS of mice.
Chapter 2 Material and Methods
Synthesis and characterization of LPEI-g-PEG copolymer

Linear polyethyleneimine HCl salt (LPEI·HCl, $M_n$ of LPEI = 17 kDa) was a kind gift from Polymer Chemistry Innovations, Inc. (Tucson, AZ). N-hydroxysuccinimidyl ester of methoxy polyethylene glycol hexanoic acid (PEG-NHS, $M_n = 10$ kDa) was purchased from NOF America Corporation (White Plains, NY). The LPEI·HCl (7.95 mg, 0.1 mmol of amine) was dissolved in 1 mL of DI water. NaOH was added to the solution drop-wise to raise the pH to 6. Then 80 mg of PEG-NHS (designed grafting degree of PEG per amine on LPEI = 8%) was added to the solution and the reaction mixture was vortexed. After incubation overnight, the reaction mixture was dialyzed against DI water and lyophilized to yield a white foam-like solid with a 95% yield. The molecular weight of the graft copolymer was characterized by GPC (gel permeation chromatography) using an Agilent 1200 series Isocratic HPLC System equipped with TSKgel G3000PWx1-CP column and TSKgel G5000PWx1-CP column (Tosoh America, Inc., Grove City, OH), which was connected with a multi-angle light scattering detector (MiniDawn, Wyatt Technology, Santa Barbara, CA). The grafting degree of PEG on LPEI was found to be 1.2%, which corresponds to an average of 4.6 PEG grafts per LPEI molecule. With designed grafting degree of 4% and 2%, the actual grafting degrees of PEG on LPEI were 0.8% and 0.6% respectively.

Particle Formulation

LPEI$_{17k}$-g-$x\%$PEG$_{10k}$ (LPEI with a MW of 17 kDa grafted with PEG: MW 10 kDa, $x$- grafting degree) is first modified with Trauts reagent (Sigma, St. Louis, MO) in the presence of dithiothreitol (DTT – Thermo Scientific, Rockford, IL). Trauts reagent is dissolved in deionized water (cell culture grade – Corning, Manassas, VA) to a concentration 2mg/ml. DTT was also dissolved in deionized water to a concentration of 20mg/ml. 9 µl of 50 mM polymer solution was mixed with 18.6 µl of Trauts reagent solution and 13 µl of the DTT solution. 59.4 µl of water was
added to this mixture and the pH is adjusted to ~7.3. The reaction is carried out for 2.5 hours. At the end of reaction the mixture was desalted, using an Amicon centrifugal filter (3k MWCO – Sigma-Aldrich), with water 3 times and reconstituted to a volume of 110ul. 10µl of DTT was added to this solution and the pH is adjusted to ~1.8-2.0. 9.8 µl of 100µM siRNA (equivalent to 16 µg of siRNA) was added to 60 µl of water. Each batch of siRNA solution was mixed with 60 µl of the polymer solution and vortexed. The nanoparticle solution is then dialyzed, against water, overnight to remove DTT and other reagents. Crosslinking was carried out for two nights with aerial oxidation similar to our previous study (Jiang, Qu et al. 2013). The nanoparticle solution was then once again dialyzed for 24 hours against water following which the samples were analyzed by dynamic light scattering (DLS), using a Malvern Zetasizer Nano ZS, which also provided information about zeta potential, followed by transmission electron microscopy (TEM). The protocols detailed above and in the following section were designed to yield an N/P ratio of 5. The volume of mixing between the polymer and siRNA was scaled accordingly to achieve additional N/P ratios. For the case of LPEI_{17k}/siRNA nanoparticle (without disulfide crosslinking) the solutions we mixed at a volume so as to produce an N/P ratio of 10.

**Particle Formulation for observing solvent polarity effect and secondary structure formation**

LPEI_{17k} - 0.6%PEG_{10k} was first modified with Trauts reagent in the presence of DTT. Trauts reagent is dissolved in deionized water to a concentration 2mg/ml. DTT was also dissolved in deionized water to a concentration of 20mg/ml. 9 µl of 50 mM polymer solution was mixed with 18.6 µl of Trauts reagent solution and 13 ul of the DTT solution. 59.4 µl of water was added to this mixture and the pH was adjusted to ~7.3. The reaction was carried out for 2.5 hours. At the end of reaction the mixture was desalted, using an Amicon centrifugal filter (3k MWCO), with water 3 times and reconstituted to a volume of 110ul. 10µl of DTT was added to this solution and the pH was adjusted to ~1.8-2.0. 163.3 µl of dimethylformamide (DMF) was added to 60 ul of the
polymer solution followed by mixing with 9.8 μl of 100μM siRNA (equivalent to 16 μg of siRNA) which yielded a 70% DMF solution. The nanoparticle solution was then dialyzed, against 70% DMF, overnight to remove DTT and other reagents. Crosslinking was carried out for 48 hours with aerial oxidation in the presence of 70%DMF. At the end of 48 hours the particles were dialyzed extensively against cell-culture grade water and then further analyzed by dynamic light scattering (DLS) and transmission electron microscopy (TEM).

Transmission electron microscopy

10 μl of nanoparticle solution, containing LPEI$_{17k}$- x%PEG$_{10k}$/siRNA, was deposited on ionized nickel grid covered by carbon. The excess liquid on the grid was pipetted out after 7 min. 6 μl of 2% uranyl acetate solution was then deposited on the grid and allowed to incubate for 30 seconds. The excess liquid was once again pipetted out and the grid was allowed to dry at room temperature prior to being examined. The samples were imaged on a Tecnai FEI-12 electron microscope.

Animal Infusion

Following anesthetization, the hair above the skull of mice was removed to expose the scalp. An incision was made along the midline to expose the skull. A hole was drilled through the skull, above the right lateral ventricle (bregma –0.5mm,1.0mm lateral). After drilling, bone fragments were cleaned away. Alzet (brain infusion kit# 3, Cupertino, CA) apparatus, as per manufacturer’s specifications, was used to place a cannula at a depth of 2.2mm. The cannula was cemented using dental cement. A long tube (FEP-tubing, SCIPRO Inc., Sanborn, NY) was used to connect the end of the cannula above the skull to a slow infusion pump (Stoelting Co. Wood Dale, IL). The tube connecting the slow infusion pump and the cannula was sufficiently long so as to allow free head and neck movement of the mice. The animal was then placed in a special enclosure – Raturn Microdialysis Stand-Alone System (with free access to food and water) where the tube going to the slow infusion microdialysis pump can be secured and the process of infusing the of
therapeutic agent was begun (0.1ul/min – during the infusion phase). At any given point there would be only one mouse present in the Raturn Microdialysis Stand-Alone System undergoing infusion (BASi, West Lafayette, IN). We used a slow infusion pump considering that we need the flexibility of having a system that would be able to deliver agents from a period varying from 2 days up to 7 days with the ability to stop infusions, as per our staggered infusion protocol (Supplementary Figure 7E). Importantly it allowed us to deliver a specific volume of therapeutic to the targeted area in the brain and to monitor the effect in the live animal which would mimic a clinical setting where the therapeutic can be potentially used. All the mice used in studies belonged to the C57BL/6J strain. In this report the right hemisphere is referred to as the ipsilateral side of the brain and the left hemisphere is referred to as the contralateral side of the brain (with reference to the side of infusion in the brain). All animal studies were performed in compliance with the Johns Hopkins Institutional Animal Care and Use Committee approved protocols.

**Immunohistochemical analysis**

Mice (controls and those undergoing infusion) were perfused with cold 4% paraformaldehyde in phosphate buffered saline (PBS). The brains were harvested and each hemisphere separated following which they were embedded in paraffin, sectioned sagitally, and processed for IHC analysis using the peroxidase-antiperoxidase method (Laird, Cai et al. 2005) with antibodies specific to BACE1 (1:500), GFAP (Dako Cytomation, Carpinteria, CA) 1:500, and Iba-1 (Wako Chemicals, Richmond, VA) 1:500. Secondary, biotinylated, goat anti rabbit antibodies were purchased from Vectashield (Burlingame, CA). The sections were counter stained with hematoxylin.

**Protein blot analysis**

Harvested mouse brains (forebrain, hippocampus, and brain stem) and spinal cords (cervical, thoracic, and lumbar) were homogenized with radioimmunoprecipitation (RIPA) buffer (Sigma)
in the presence of protease inhibitors (Thermo Scientific, Rockford, IL), centrifuged, and the supernatant was used for western blotting. The lysates were run on a 4-12% bis-tris gel (Life Technologies, Grand Island, NY), and then transferred on to a PVDF membrane. For western blot analysis, the membranes were blocked in 5% milk in TBS-T for an hour and then probed with antibodies specific for BACE1 (1:2500), GAPDH (1:20000, Sigma), APP-CTF (1:8000), and Actin (1:5000, Sigma). Band densitometry analysis was performed using Image Studio Lite software from LI-COR (Lincoln, NE).

**In vitro knockdown and cell viability studies**

The siRNAs used in this study are shown in the table below.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>BACE33:</td>
<td>Sense- 5'-GAGCCCUCUUUGACUCCCUUGUGA</td>
</tr>
<tr>
<td></td>
<td>Antisense- 5'-UCACCAGGGAGUCAAAGAAGGGCUC</td>
</tr>
<tr>
<td>BACE23:</td>
<td>Sense-5'-GAGGGAGCAUGAUCAUUGGUUGUAAU</td>
</tr>
<tr>
<td></td>
<td>Antisense- 5'-AUACCACCAAUGAUCAUGCUCCCUC</td>
</tr>
<tr>
<td>APP35:</td>
<td>Sense-5'-GCUGGAUGGAUSUUUGUGAGACCCAU</td>
</tr>
<tr>
<td></td>
<td>Antisense- 5'-AUGGGUCUCACAAACAUCCAUCCGC</td>
</tr>
<tr>
<td>APP34:</td>
<td>Sense-5'-GACCGAGGUUCUGGCGAGCAAAACAU</td>
</tr>
<tr>
<td></td>
<td>Antisense- 5'-AUGUUUGUCAGCCCAGAACCUGGUC</td>
</tr>
<tr>
<td>APP33:</td>
<td>Sense- 5'-UCAGGAUUUGAAGUCCGCAUAAAA</td>
</tr>
<tr>
<td></td>
<td>Antisense- 5'-UUUGAUGGCGACUUCAAAUCUGA</td>
</tr>
</tbody>
</table>

Luciferase Stealth control and fluorescently labeled (Alexa-555) sequences were all purchased from Life Technologies. Lipofectamine2000 in Opti-Mem media was used for transfection as per manufacturer recommendations (Life Technologies). Transfection experiments were performed
in a 6-well plate with N2a cells maintained in 10%FBS, 1× Glutamax, 1× MEM-NEAA, 1× sodium pyruvate and antibiotic free conditions (Life Technologies). Cells were transfected with siRNA packaged in nanoparticles or Lipofectamine2000. Cell culture medium was replaced after 16 hours with fresh medium and the cells were harvested for further analysis after 24 hours. For harvesting cells, the media was first aspirated from each well. Following which, each well was washed twice with cold (4°C) phosphate buffered saline. A cell scraper was used to collect the cells from each well and protein extraction was performed using the RIPA buffer following a similar protocol as the tissue protein extraction. The prepared lysates were further probed for protein content via western blot analysis. Cell viability was checked via an MTT assay based on the manufacturer recommended protocol (Life Technologies). In a 96-well plate N2A cells were treated at a scale proportional to that of a transfection in a 6-well plate. A similar time scale of incubation for 24 hours was maintained after which time knockdown analysis was performed. The cytotoxicity of the samples was analyzed on an EPOCH BIOTEK (Winooski, VT) plate reader.

**Confocal studies**

Mice (with neurons sparsely expressing GFP, Thy-1 Promoter, gift from the Richard Huganir lab at Johns Hopkins) were perfused with cold 4% paraformaldehyde in phosphate buffered saline (PBS), 24 hours after undergoing infusion protocol. The brains were harvested and each hemisphere separated. Following which they were post fixed 4% paraformaldehyde for 24 hours. The tissues were then treated for 48 hours with 30% sucrose for cryopreservation following which they were placed in a mold with OCT and prepared for cryosectioning. The tissue were sectioned sagitally (16 µm thickness), and collected on superfrost glass slides and stained with antibodies specific to BACE1 (1:500), GFAP (Dako Cytomation), and Microglia (Wako Chemicals). Mounting media (Life Technologies), preloaded with DAPI, was applied prior to application of the cover slip. Microscopic analyses were performed on a Zeiss LSM 510 microscope. In the case
of cell culture, cells were grown on a cover slip placed at the bottom of a 6 well plate. At the end of the transfection study, with fluorescently labeled siRNA, over the same conditions as the knockdown studies, the cover slip was washed in PBS, and 0.2M acetic acid in 0.5M sodium chloride solution. Following which the cover slips were treated with 0.4% trypan blue solution Hanks buffered salt solution to quench extracellular fluorescence (Rejman, Oberle et al. 2004). Mounting media (Life Technologies), preloaded with DAPI, was applied to a glass slide prior to placing the coverslip on it for further microscopic analysis.

**Gel retardation assay**

We used a 2% agarose gel (with ethidium bromide) in a TAE buffer to elucidate release of siRNA from micellar nanoparticle formulations. Nanoparticle formulations were incubated with varying concentrations of dextran sulfate following which 20ul of each sample was loaded in each well with loading buffer. Samples were run on the gel for 20 mins at 120V.

**Statistical analysis**

Statistical comparisons were performed using ordinary one way analysis of variance (ANOVA). Comparisons between groups were performed using Tukey’s multiple comparisons test using GraphPad Prism Software (La Jolla, CA). Error bars used in this study are in SD and SEM and appropriately noted in the figures.
Chapter 3 Results
To tune the shape of siRNA loaded micellar nanoparticles, we grafted LPEI$_{17k}$ with varying degrees of PEG$_{10k}$ (as a condensing agent). Despite siRNA packaging and transfection efficiency occurring with formulations at higher N/P ratios (i.e., molar ratio of amines in the polymer backbone to phosphates in the siRNA) (Shim and Kwon 2009), we chose to dissolve and mix both polymer and siRNA in water at a relatively lower N/P ratio of 5, so as to limit cytotoxic effects (*in vitro* and *in vivo*) (Zheng, Pavan et al. 2012). To enhance the stability of these shaped nanoparticles and improve delivery of their payload in the cytoplasm of cells, we used a disulfide crosslinking strategy (Lee, Green et al. 2009, Jiang, Zheng et al. 2010). Under these conditions we observed, using transmission electron microscopy (TEM), that nanoparticles made with these polymers of increasing PEG grafting density of 0.6%, 0.8% or 1.2% displayed a propensity to form respectively, worm-like, rod-like, or spherical morphologies (Figs. 1a-c). While the non-crosslinked, non-PEGylated LPEI$_{17k}$ particles exhibited marked size increase as measured by DLS and were prone to aggregate under physiological conditions, the size of disulfide-crosslinked, PEGylated particles which did not aggregate were within the 100nm range (Fig. 1d). Taken together with the observation that non-PEGylated disulfide-crosslinked particles also formed aggregates (Fig. 2), our findings suggest that the condensation driven by the PEG on the LPEI backbone is essential for formation of siRNA loaded nanoparticles and their morphology, a determinant that may impact on *in vivo* delivery of the payload.

We then investigated whether the tuning of the shape of nanoparticles with LPEI$_{17k}$-x%PEG$_{10k}$ to obtain different morphologies with the same starting backbone might be amenable for delivering siRNA against different targets. Since minor deviations in N/P ratio at lower range values can influence stability of nanoparticles and impact on their delivery of the siRNA payload (Zheng, Pavan et al. 2012), we synthesized particles using LPEI$_{17k}$-0.6%PEG$_{10k}$ with N/P ratios which varied from 3 to 6. Each particle formulation was made independently, except for the differing ratios of the polymer to the siRNA in the initial mixing phase; all particles were stabilized with
disulfide crosslinking. Importantly, we observed that as the N/P ratio increased from 3 to 6, the particles transitioned from a worm-like to a rod-like morphology at N/P of 6 (Fig. 3).

As we previously have elucidated the effect of solvent polarity on the formation of nanoparticles encapsulating plasmid DNA (Jiang, Qu et al. 2013), we asked whether such an effect can be observed with siRNA loaded nanoparticles. When the initial particle formation conditions was changed from water to a mixture of water:DMF (3:7(v/v)), we observed that the nanoparticles possessed a short rod morphology as opposed to the worm-shaped particles made in pure water. After dialyzing the particles against water followed by incubation of particles in water for two weeks at room temperature, we observed that these nanoparticles again exhibited the worm-shaped morphology (Fig. 4), suggesting that the worm-shaped morphology was possibly a secondary structure derived from the initial particle formation step. We also noted that nanoparticles were able to maintain their worm shaped morphology even after incubation for three months (Fig. 5). Thus, we have developed a simple method to tune the shape of siRNA loaded nanoparticles by varying the amount of PEG grafted onto the linear polymer to drive their condensation.

Before assessing the influence of the shape of nanoparticles for delivery of siRNA to knockdown targets of interest in the CNS, we screened for the appropriate siRNA sequences and evaluated the ability of our siRNA loaded nanoparticles to knockdown BACE1 and APP in a cell culture model, namely, N2a cells which endogenously express both genes (Li, Zhou et al. 2006). We first verified that the LPEI_{17k} complexed with siRNA against either BACE1 or APP was capable of forming nanoparticles that were below 100nm in size (Fig. 6). After screening multiple siRNAs targeting either BACE1 or APP by transfection into N2a cells, we selected two top candidates, BACE33 and APP35, for further evaluation (Fig. 7). Protein blot analysis revealed that while naked siRNAs were unable to alter the protein levels of their targets, LPEI_{17k} encapsulating BACE33 or APP35 reduced the level of BACE1 or APP, respectively, by 63.3 ± 25.4% or 75.6 ± 10.2% of control (Figs. 8a-c). Note that the knockdown of BACE1 by BACE33 did not alter the
level of APP and neither did the knockdown of APP by APP35 affect the level of BACE1. We showed that such nanoparticles can deliver the siRNA into the cytoplasm of cells (Fig. 9a).

Moreover, transfections performed with LPEI\textsubscript{17k}/siRNA nanoparticles at a maximum dose of 5ug of siRNA did not elicit cytotoxicity as judged by the viability of transfected cells (Fig. 9b). Empirically, we determined that an optimal dose for knockdown of BACE1 is between 3 to 5 ug siRNA (Fig. 9c). Together, these results suggest that this nanoparticle system is efficient in its cargo delivery capability and suitable for further in vivo investigation.

To assess the influence of the shape of nanoparticles in the delivery of siRNAs to cells of the CNS globally, we performed intraventricular infusion of differentially shaped nanoparticles in awake and freely moving mice (Fig. 10a), a delivery approach thought capable of achieving global distribution of payload in the brain (Wang, Ghosh et al. 2008, Yu, Pendergraft et al. 2012). Mice were initially infused with up to 64 µg of siRNA in the right lateral ventricle (referred as the ipsilateral side) over a 7-day period (Fig. 10b). Unfortunately, such effort of using our optimized formulations from the in vitro studies did not lead to reduction in levels of BACE1 (Fig. 11).

Consistent with other studies (Williford, Wu et al. 2014), we also noted toxicity associated with the higher N/P ratios of nanoparticle formulations. Consequently, we decided to evaluate the efficiency of delivery of siRNA utilizing LPEI\textsubscript{17k-x%PEG\textsubscript{10k}}, stabilized with disulfide crosslinking. We hypothesized that the increased half life and stealth properties of PEG would provide a better opportunity for siRNA delivery to cells of the CNS. We also used a low N/P ratio of 5 so as to mitigate toxicity in the CNS that would be counterproductive towards any gain in transfection efficiency. We showed that, in an N2a cell culture model, for all of the LPEI\textsubscript{17k-x%PEG\textsubscript{10k}}/siRNA nanoparticles the viability was greater than 95% when compared with the untransfected control (Fig. 9b). Importantly, as noted earlier, we were able to develop distinct nanoparticles that were worm-like, rod-like and spherically shaped, which presented us a unique opportunity to test the siRNA delivery efficacy to the CNS using these shaped particles. Testing the knockdown efficiency, of these LPEI\textsubscript{17k-x%PEG\textsubscript{10k}}/siRNA nanoparticles in vitro was
challenging due to the lack of transfection in cells on account of the PEG moiety (Mishra, Webster et al. 2004).

To determine whether nanoparticles can be transported into the brain parenchyma, we followed in brains of mice, intraventricularly infused for two days, the fate of fluorescently labeled siRNA encapsulated in nanoparticles. To facilitate identification of neurons in the brain, we used mice genetically encoded with GFP in which neurons are sparsely marked by GFP in the cytoplasm (Feng, Mellor et al. 2000). Proximal to the lateral ventricle, we observed a gradient of fluorescently labeled siRNA emanating from the infusion site towards the brain parenchyma (Fig. 12). Importantly, the accumulation of the siRNA with the nuclei (DAPI) strongly suggests that these nanoparticles were able to gain access to the cytoplasm of cells in the brain parenchyma.

To assess potential untoward side effects of nanoparticles infused into brains of mice up to one week, we examined glial cells which are normally activated and migrate to the site of injury in the brain (Holguin, Frank et al. 2007). Since cannulation of the right lateral ventricle would lead to the injury of tissue in the immediate vicinity, we first confirmed that similar activation of glial cells at the injury site occurred for animals infused with vehicle or those with nanoparticles (Fig. 13). Note that in regions away from the site of injury, a decrease in glial cell activation was observed (Figs. 13a-c, d-f). Importantly, no difference in glial cell activation was seen in the contralateral side of brains between both sets of mice. While these results confirm that these nanoparticles do not illicit an untoward response over a two day period, we also examined any potential impact for mice infused for at least 7 days. Using markers of astrocytic and microglial activation, we failed to reveal any difference in activation of both cell types in the hippocampus of mice infused with the distinctly shaped nanoparticles as compared to control (Figs. 14,15). Taken together with the observation that microglial and astrocytic cells continued to maintain their normal morphology, our findings suggest that these shaped nanoparticles do not illicit an unfavorable response in the brain when infused over one week.
Importantly, while worm and sphere shaped nanoparticles did not alter BACE1 levels, infusion of rod shaped nanoparticles into the lateral ventricle led to a robust knockdown to 36.8 ± 4.8% or 42.8 ± 2.9% in the ipsilateral or contralateral side of the cortex, respectively, as compared to the vehicle infused control (Figs. 16a-c). However, we observed reduction of BACE1 levels by 33.3 ± 6.7%, 24.1 ± 4.1%, or 38.2 ± 3.4% with worm, sphere, or rod shaped nanoparticles, respectively, in the ipsilateral hippocampus (Fig. 17). In the contralateral hippocampus we observed a knockdown of 24.2 ± 4.5%, 28.9 ± 4.1%, or 35.6 ± 5.9% in BACE1 levels using respectively, worm, sphere, or rod shaped nanoparticles (Fig. 17). Together, these findings indicate that the shape of nanoparticles is one determinant that regulates their efficiency for delivery to cells in the CNS. To evaluate how extensive these nanoparticles could distribute throughout the CNS, we examined their ability to knockdown BACE1 in the brainstem and spinal cord, regions that are farther away from the site of infusion. Importantly, we too observed that our nanoparticles were able to significantly reduce levels of BACE1 in the brainstem and spinal cord of mice (Figs. 18,19). Together, our findings thus establish that efficient siRNA delivery to cells throughout the central nervous system is accomplished by shaped nanoparticles based on the LPEI\textsubscript{17k}-x\%PEG\textsubscript{10k} platform.
Chapter 4 Discussion
That siRNA can mediate selective mRNA degradation to modulate levels of specific proteins has fueled therapeutic efforts since its initial discovery to develop suitable carriers to efficiently package and deliver siRNA to cells/organs (Kanasty, Dorkin et al. 2013). While this remains to be a great challenge, tremendous progress has been made towards developing suitable carriers for siRNA as therapeutics which in the form of nanoparticles have reached clinical trials (Coelho, Adams et al. 2013). Our recent work showing that the shape of DNA loaded nanoparticles is a determinant of transfection efficiency in a rat liver model (Jiang, Qu et al. 2013) supports the notion that the shape of nanoparticles (Kolhar, Anselmo et al. 2013) could be important for productive interaction of nanoparticles with target cells. Pioneering work involving the use of DNA as building blocks have been extended to RNA and more recently to long RNAi (microsponges) that were developed in an effort to overcome challenges of packaging short pieces of siRNA (Lee, Lytton-Jean et al. 2012, Shopsowitz, Roh et al. 2014). These RNA species when encapsulated by a polymer typically formed spherical particles (the thermodynamically favored state). While shape control using RNA as a building block has been achieved (Afonin, Kireeva et al. 2012), the control of nanoparticle shape with siRNA as the payload remains elusive. Our finding that a variety of siRNA nanoparticle shapes using LPEI-g-PEG copolymer carriers establishes the first evidence that the shape of polycation/siRNA micellar nanoparticles can be varied in a systematic fashion. That increasing the hydrophilic content of an amphiphilic block polymer changed the shape of self-assembled micelles from worms to spheres (Rajagopal, Mahmud et al. 2010) led us to hypothesize that, in contrast to the observation from molecular dynamics simulations that DNA drives shapes of DNA nanoparticles (Jiang, Qu et al. 2013), these various shapes observed with siRNA micellar nanoparticles is driven by the polymer backbone with varying degree of grafting of PEG. By exploiting the advantage of the delivery capability of siRNA by nanoparticles at low N/P ratios (Zheng, Pavan et al. 2012), we successfully tuned the shape of siRNA nanoparticles by modestly changing the N/P ratio (reflecting a relative alteration in PEG content). An added advantage of
working at such a low N/P ratios is the mitigation of cytotoxic effects that have been reported for formulations with higher N/P ratios (Fukushima, Miyata et al. 2005). Importantly, our approach of simple mixing of components that assemble into distinct shapes would lend itself to scalable settings and high throughput analyses. Although we favor the idea that the long worm shaped species represent a secondary structure derived from short rods, future molecular dynamic studies will be instrumental to clarify key thermodynamic factors driving the formation of these distinct siRNA nanoparticle morphologies. This study confirmed that increasing the PEG grafting density leads to more condensed spherical morphology, whereas lower grafting density yields rod- and worm-like micelles. This observation can be explained using the traditional micelle packing models for amphiphilic diblock copolymer micelles assembled in aqueous media (Israelachvili 2011). Assuming that the degrees of condensation between the LPEI backbone and siRNA are similar for graft copolymers with different PEG grafting densities under the same N/P ratio, since the grafting densities are relatively low (< 2%), condensation of siRNA with copolymers having a higher PEG grafting density generates micellar nanoparticles with a higher PEG surface density, thus favoring the formation of spherical micelles. This is analogous to micelle assembly, where the shape control is governed by the volume ratio of the hydrophilic (corona) to hydrophobic (core) blocks—higher ratio favors spherical micelle formation and lower ratio yields worm-like micelles (Israelachvili 2011). The trend observed by varying N/P ratio is also consistent with this interpretation: a higher N/P ratio leads to higher PEG density on micelle surface, favoring spherical micelle formation, although it is surprising that such a small range of N/P ratio variation from 3 to 6 can lead to significant shape variation.

Studies have highlighted challenges involved in designing a suitable method to target the key enzymes involved in the APP pathway (Menting and Claassen 2014, Reiman 2014). Since mechanism based toxicities are associated with inhibition of γ-secretase (Li, Wen et al. 2007), we focused on developing a suitable delivery vehicle for siRNA targeting BACE1 and APP. Previously, we and others employed RNAi strategies delivered by viral vector to reduce levels of
BACE1, which emerged as a powerful tool to deliver short hairpin RNA to decrease protein levels (Laird, Cai et al. 2005, Singer, Marr et al. 2005). An alternative approach to regulate levels of BACE1 is the RVG-exosomes system (Alvarez-Erviti, Seow et al. 2011). To mitigate putative safety concerns, such as insertional mutagenesis and the risk of toxicity associated with viral vectors (Kamat, Shmueli et al. 2013), we used another non-viral method for siRNA delivery, namely the LPEI base polymer system which has been shown to be less toxic as compared to the traditional branched PEI platform (Bonnet, Erbacher et al. 2008). Indeed, we confirmed the lack of cytotoxicity of this nanoparticle system as judged by the lack of astrocytic or glial cell activation in response to nanoparticle infusion over a 7 day period. After validating in a cell culture model the LPEI17k system to deliver siRNA to modify levels of BACE1 and APP, two different proteins in the APP pathway of AD, we evaluated their efficacy in the CNS of mice. Since AD is a neurodegenerative disorder that globally affects the brain, we focused on ensuring that the nanoparticles were circulated throughout the brain as opposed to a local infusion close to the site of interest. In contrast to several studies focused on inducing RNAi in the CNS of mice (Passini, Bu et al. 2011, Lima, Prakash et al. 2012), our discovery of shaped nanoparticles provides the opportunity to evaluate whether shape of nanoparticles is a major determinant for efficient delivery of siRNA throughout the CNS. Our proof of principle studies established that while worm and sphere shaped nanoparticles did not alter its levels, rod shaped nanoparticles significantly reduced BACE1 levels in the ipsilateral and contralateral side of the brain, indicating that rod shaped nanoparticles represent superior delivery vehicles for siRNA in the CNS. Consistent with this view is the demonstration that rod shaped particles decorated with antibody ligands accumulated preferentially in the brain, although a distinction as to whether such nanoparticles were restricted to the neural vasculature or gained access to the brain parenchyma was not established (Kolhar, Anselmo et al. 2013). Interestingly, that the reduction in BACE1 levels observed in the hippocampi were not significantly different amongst various shaped nanoparticles could be attributed to the proximity of the hippocampus to the lateral ventricles.
Moreover, our findings of knockdown in the brain stem and spinal cord, regions that are far from the infusion site, further support the ability of both worm and rod shaped particles to achieve global distribution and efficient delivery of siRNAs. Although we did not see a robust knockdown in the thoracic spinal cord, future studies using intrathecal infusion of these shaped nanoparticles should clarify differences in the efficiency of knockdown in the spinal cord with this nanoparticle system. Further exploration of attributes of the rod shaped particles that promote greater efficacy should allow us to design more suitable carriers to enhance CNS delivery.

Although we have shown here that rod shaped particles work best in the CNS of mice, it remains to be demonstrated as to whether the worm and sphere shaped particles have applications in other cells/organs. That micron sized worm shaped particles have better circulation properties in rats and worm as compared to spherically shaped particles target the liver more efficiently (Geng, Dalhaimer et al. 2007, Jiang, Qu et al. 2013) would support this notion. Targeting ligands against the insulin receptor, transferrin receptor or those such as cell penetrating peptides have been established as valuable tools to improve delivery of cargo to cells of interest (Kamide, Nakakubo et al. 2010, Atwal, Chen et al. 2011).

In conclusion, we have shown here that micellar nanoparticles with worm- and rod-like, and spherical shapes can be prepared by self-assembly of the complexes between siRNA and LPEI-g-PEG copolymer carriers. The PEG corona and reversibly crosslinked core of the micelles enable these nanoparticles to be stable under physiological conditions. Interestingly, these micellar nanoparticles revealed differences in knockdown capability following infusion into the lateral ventricles in mice with the rod-like micelles showing the most effective and selective knockdown of a key therapeutic target in AD. Future development of siRNA delivery strategies leveraging shape as a tunable parameter would broaden the toolkit we have at our disposal for creating a translatable platform for RNAi therapeutics.

**Figures**
Figure 1. Tuning the shape of LPEI-g-PEG/siRNA micellar nanoparticles by varying PEG grafting degree. (a) LPEI_{17k}-g-0.6%PEG_{10k}/siRNA nanoparticles (N/P=5) showing worm-like morphology. (b) LPEI_{17k}-g-0.8%PEG_{10k}/siRNA nanoparticles (N/P=5) showing rod-like morphology. (c) LPEI_{17k}-g-1.2%PEG_{10k}/siRNA nanoparticles (N/P=5) showing spherical morphology. All scale bars = 200 nm. (d) Particle size measured by DLS in water and 150 mM NaCl after incubation at room temperature for 4 h (n ≥ 3, Mean ± SD). The LPEI_{17k}/siRNA nanoparticles without PEG grafts (0%) were prepared at an N/P ratio of 10 without crosslinking.
Figure 2. Transmission electron microscopy (TEM) images of non-PEGylated crosslinked LPEI_{17k}/siRNA particles at N/P ratio of 5, indicating severe aggregation of complexes in 150 mM NaCl. Panels (a) and (b) depict two different magnifications of the crosslinked samples as analyzed by TEM with scale bars of 400 nm and 2 µm, respectively.

We prepared crosslinked nanoparticles using non-PEGylated LPEI_{17k} using a similar protocol as compared to other formulations (see Methods section). When compared with the PEGylated crosslinked nanoparticles that display distinct morphologies, we observed a formation of significantly aggregated species. This suggests that the PEG grafts on the LPEI backbone plays an important role towards stabilizing the formation of nanoparticles, with various morphologies, encapsulating siRNA.
Figure 3. TEM images showing effect of N/P ratio on the shape of LPEI$_{17k}$-g-0.6%PEG$_{10k}$/siRNA micellar nanoparticles. (a-d) LPEI$_{17k}$-g-0.6%PEG$_{10k}$/siRNA nanoparticles can be varied from long worms to short rods by adjusting the N/P ratio from 3 to 6. Scale bars = 200 nm.

Using LPEI$_{17k}$-g-0.6%PEG$_{10k}$ as a gene carrier, we prepared crosslinked siRNA-loaded micellar nanoparticles following a similar protocol as compared to other formulations (see Methods section), while varying only the N/P ratio from 3 to 6. These results, depicting a transition from long worm-like to short rod-like nanoparticles, confirm that subtle variations in N/P ratios can have a significant impact on particle morphology.
Figure 4. Worm shaped particles being retrieved from short rod shaped particles that were made in the presence of 70% DMF. (a) LPEI_{17k-0.6%PEG_{10k}}/siRNA nanoparticles, N/P 5, short rods, made in 70%DMF. Scale bar 200 nm. (b) LPEI_{17k-0.6%PEG_{10k}}/siRNA nanoparticles, N/P 5, worms, after two weeks. Scale bar 400 nm.

Switching from DI water to 70% DMF, in the initial phase of the particle formation step, results in a morphology similar to that of short rods as opposed to worms in the previous case. It is important to note here that 70% DMF conditions were maintained only during the initial phase of particle formation and crosslinking steps following which the particles were dialyzed extensively against cell culture grade DI water. After incubating the particles in water for two weeks we performed TEM again on the dialyzed samples and observed that we once again obtain a worm like morphology similar to the case of making particles in purely DI water.
Figure 5: LPEI_{17k}-2\%PEG_{10k}/siRNA nanoparticles, N/P 5, worms, stable after 3 months.

Scale bar 200nm.

For long-term and wide scale applicability of nanoparticles as a vehicle for delivery of therapeutic siRNA it would be advantageous if they were stable and can be easily stored. We assessed the architecture of our particles by incubating them at room temperature (or 4° C) for 3 months. We can appreciate that the worm like particle morphology was still intact.
Figure 6. Gel retardation and zeta potential analyses of LPEI_{17k} g-PEG_{10k}/siRNA micellar nanoparticles, and TEM image of LPEI_{17k}/siRNA nanoparticles. (a) Release of free siRNA from LPEI_{17k} g-PEG_{10k}/siRNA nanoparticles in the presence of 20 µM DS. DS-Dextran Sulfate, FS-Free siRNA. (b) Release of siRNA from the same particles when challenged with 150 µM DS. (c) TEM analysis of nanoparticles formed by the complexation of LPEI_{17k} with siRNA (N/P = 10). Scale bar = 100 nm. (d,e) Zeta potential measurements of nanoparticles encapsulating siRNA formed using LPEI_{17k} g-PEG_{10k} and the non-PEGylated LPEI_{17k} in water (d) and PBS (e) (n = 3, Mean ± SD, ANOVA, n.s.–between the negatively charged complexes).

Gel retardation assay results show the release of free siRNA from LPEI_{17k} g-1.2%PEG_{10k}/siRNA nanoparticles, which was enhanced in the presence of 20 µM DS (Fig. S3a). Under similar conditions the remaining formulations such as LPEI_{17k} g-0.8%PEG_{10k}/siRNA, LPEI_{17k} g-0.6%PEG_{10k}/siRNA, and LPEI_{17k}/siRNA display a stronger interaction between the polymer and siRNA, as evidenced by the lack of siRNA running down the gel (Fig. S3a). The same particles when challenged with 150 µM DS now release siRNA that we observe migrating down the gel (Fig. S3b). Zeta potential measurements indicate a slightly negatively charged particle for siRNA encapsulated with LPEI_{17k} g-PEG_{10k}, as opposed to positively charged particles formed when LPEI_{17k} is used to encapsulate siRNA. To further investigate the zeta potential values under physiological conditions, so as to get a better understanding of the attributes the particles might have in vivo, we measured the zeta potential in PBS (Fig. S3e). We observed a drop in the zeta potential values when comparing the charge of the nanoparticles made with LPEI (N/P 10) as measured in water. We also observed a decrease in zeta potential to almost half its value when the particles are made at N/P 5. Additionally, when measured in PBS, comparing zeta potential of these the non-PEGylated particles with the PEGylated we observe that they are close to neutral charge.
Figure 7. siRNA sequence validation via *in vitro* knockdown studies. siRNA sequences targeting BACE1 and APP were validated via transfection with Lipofectamine (Lipo). Sequences show a selective and robust knock down effect.
**Figure 8. In vitro knockdown efficiency of LPEI\textsubscript{17k}/siRNA nanoparticles in N2a cells.**

(a) Protein blot analysis of *BACE1* and *APP* levels after N2a cells were transfected with nanoparticles prepared with sequences BACE33 and APP35, respectively, or with Lipofectamine (positive control) and naked sequences (negative control). (b-c) Quantification of protein blot analysis of (b) *BACE1* and (c) *APP* protein levels as compared to non-transfected N2a cells (n = 4, Mean ± SEM, ANOVA, F = 19.75, p< 0.0001). All *in vitro* studies were performed at an N/P ratio of 10.
Figure 9. *In vitro* knockdown studies of LPEI$_{17k}$/siRNA nanoparticles in the context of N2a cells.

(a) Microscopic analysis of the *in vitro* cell culture model confirmed that fluorescently labeled siRNA (Red) was delivered to the cytoplasm of cells using LPEI$_{17k}$ (nuclei-DAPI). We noted the classical pattern of siRNA accumulation in the cell around the nucleus (stained blue). (b) MTT assay analysis confirms that over the transfection period of 24 h, the various formulations built on the LPEI$_{17k}$ platform were not toxic ($n = 5$, Mean ± SEM, ANOVA, not significant--n.s.). (c) Transfection studies in N2a cells with varying amounts of siRNA delivered in the form of nanoparticles with the LPEI$_{17k}$. The first three lanes are a dilution series of protein lysates from untransfected cells.
Figure 10. Schematic showing the infusion setup and dosing regimen of micellar nanoparticles in mice. (a) Infusion setup allowing for continuous infusion into the lateral ventricle of the brain of a mouse. The tube from the cannula was connected to a slow infusion pump. The animals were awake, freely moving, and had free access to food pellets and water. (b) Schematic of infusion regimen. All infusions were performed at 0.1 µl/min.
**Figure 11.** Protein blot analyses detailing examples of initial studies involving siRNA nanoparticles being infused into the brain of mice. All infusions were performed in the right (ipsilateral) lateral ventricle at a dose of 16 µg of siRNA, complexed in nanoparticle form, per day, with the same dosing regimen (shown in Fig. 3b). FB-Forebrain, NP-Nanoparticles, H-Hippocampus. (a-c) Protein blot analyses of BACE1 in the forebrain and hippocampus, following infusion with uncrosslinked LPEI_{17k}/siRNA nanoparticles (a), crosslinked LPEI_{17k}/siRNA nanoparticles (b), uncrosslinked LPEI_{17k-g-0.6%PEG_{10k}}/siRNA micellar nanoparticles (c) in the lateral ventricle of the brain.

We observed that BACE1 levels remain unaltered in both the ipsilateral and contralateral side of the brain of mice undergoing infusion with uncrosslinked LPEI_{17k}/siRNA nanoparticles, crosslinked LPEI_{17k}/siRNA nanoparticles, or uncrosslinked LPEI_{17k-g-0.6%PEG_{10k}}/siRNA nanoparticles. These results underscore the importance of using crosslinked micellar nanoparticles with varying morphology towards knockdown of BACE1 protein levels in a global manner in the CNS.
**Figure 12. Microscopic analysis of fluorescently labeled siRNA encapsulated in micellar nanoparticles in the brain parenchyma.** Animals were infused with micellar nanoparticles in the lateral ventricle for two days prior to harvesting the brain tissue. (a) Fluorescently labeled siRNA (red) is observed in the brain parenchyma, proximal to the lateral ventricle infusion site. DAPI (nuclei) and neurons (green). Scale bar = 20 µm.

To assess whether nanoparticles encapsulating siRNA can be transported in the brain parenchyma of mice, we infused nanoparticles encapsulating fluorescently labeled siRNA (red) in the brains of mice that were endogenously expressing GFP that sparsely labeled neurons. Upon inspection closer to the infusion site, we observed a gradient in the siRNA signal as we tracked the siRNA moving farther away from the infusion site. The fluorescence pattern of siRNA (red) with the DAPI (nuclei) and neurons (green) suggests that the siRNA was able to access the cytoplasm of cells in the brain parenchyma.
Figure 13. Microscopic analysis of astrocytic cells (GFAP+ staining) in response to brain infusion over 2 days. (a–c) vehicle infusion, (d–f) nanoparticle infusion (16 µg of encapsulated siRNA/day).

(a,d) Activated glial cells migrating towards the site of injury proximal to the lateral ventricle infusion site. (b,e) Gradient in glial cell activation and migration towards the site of injury. (c,f) Glial cell activation moving farther away from the site of infusion. Scale bar = 20 µm in (d), for the remaining panels 100 µm.

Utilizing GFAP staining, we evaluated any untoward astrocytic response over a short time frame of 2 days. At the infusion site, we observe a strong activation of astrocytic cells in the case of both vehicle and nanoparticle infused brains (a, d). Moving farther away from the infusion site, we observed a gradient in the glial cell activation (b, e) to the point that sufficiently far away from the infusion site there is no cell activation (c, f). Taken together, these data suggest that the infusion of the nanoparticles based on our current platform does not elicit an astrocytic response over an acute timeframe.
Figure 14. Microscopic analysis of astrocytic and microglial activation in the contralateral hippocampus as assessed by immunohistochemistry after infusion of micellar nanoparticles containing 64 µg of siRNA following a 7-day infusion protocol. (a–d) No significant difference was observed in astrocytic (GFAP staining) response in the contralateral hippocampus when infused with vehicle (a), worm-like nanoparticles (b), spherical nanoparticles (c), and rod-like nanoparticles (d). (e–h) No significant difference was observed in microglial (Iba-1 staining) response in the contralateral hippocampus when infused with vehicle (e), worm-like nanoparticles (f), spherical nanoparticles (g), and rod-like nanoparticles (h).

Scale bar = 200 µm; Inset scale bar = 20 µm.
Figure 15. Microscopic analysis of astrocytic and microglial activation in the ipsilateral hippocampus as assessed by immunohistochemistry after infusion of micellar nanoparticles containing 64 µg of siRNA following a 7-day infusion protocol. (a–d) No significant difference was observed in astrocytic (GFAP staining) response in the ipsilateral hippocampus when infused with vehicle (a), worm-like nanoparticles (b), spherical nanoparticles (c), and rod-like nanoparticles (d). (e–h) No significant difference was observed in microglial (Iba-1 staining) response in the ipsilateral hippocampus when infused with vehicle (e), worm-like nanoparticles (f), spherical nanoparticles (g), and rod-like nanoparticles (h). Scale bar = 200 µm; Inset scale bar = 20 µm.

While we previously evaluated the glial cell response over a 2-day infusion protocol, it was essential to assess the response of these cell types over the 7 day infusion protocol to evaluate the safety profile of the various nanoparticles that we were infusing in the lateral ventricles of the mice. The lack of distinguishing attributes in the context of glial cell activation and their morphology, when comparing nanoparticle infused brains with vehicles infused brains, further attests to the lack of cytotoxicity induced in the brains of mice when infused over a week.
Figure 16. *In vivo* BACE1 knockdown in the cortex of mice infused with worm-like, rod-like, and spherical micellar nanoparticles. All infusions were performed in the right (ipsilateral) lateral ventricle at a dose of 16 µg of siRNA/day with the same dosing regimen for all nanoparticles as show in Fig. 3b (n = 4 for infusion of worm-like, rod-like and spherically shaped nanoparticles, scrambled and naked siRNA infusion studies were performed in duplicate). (a) Protein blot analysis of BACE1 levels in the cortex in both the right (ipsilateral) and left (contralateral) hemispheres after delivery of sequence BACE33 using worm-like (W), spherical (S) and rod-like (R) nanoparticles. KO: BACE knockout, Co: vehicle infusion, Sc: Scrambled siRNA complexed with LPEI17k-g-0.8%PEG10k, N: naked siRNA sequence BACE33. (b,c) Quantification of BACE1 levels from protein blot analysis in the (c) ipsilateral cortex (Mean ± SEM, ANOVA, F = 9.133, p< 0.05, Tukey’s multiple comparisons test), and (d) contralateral cortex (Mean ± SEM, ANOVA, F = 17.91, p< 0.0001, Tukey’s multiple comparisons test).
Figure 17. *In vivo* BACE1 knockdown in the hippocampi of mice infused with worm-like, rod-like, and spherical micellar nanoparticles. All infusions were performed in the right (ipsilateral) lateral ventricle at a dose of 16 µg of siRNA/day with the same dosing regimen for all nanoparticles as show in Fig. 3b (n = 4 for infusion of worm-like, rod-like and spherically shaped nanoparticles, scrambled and naked siRNA infusion studies were performed in duplicate). (a) Protein blot analysis of BACE1 levels in the hippocampus (b) in both the right (ipsilateral) and left (contralateral) hemispheres after delivery of sequence BACE33 using worm-like (W), spherical (S) and rod-like (R) nanoparticles. KO: BACE knockout, Co: vehicle infusion, Sc: Scrambled siRNA complexed with LPEI_{17k}-g-0.8%PEG_{10k}, N: naked siRNA sequence BACE33. (b,c) Quantification of BACE1 levels from protein blot analysis in the (b) ipsilateral hippocampus (Mean ± SEM, ANOVA, F = 13.45, p< 0.0001, Tukey’s multiple comparisons test), and (c) contralateral hippocampus (Mean ± SEM, ANOVA, F = 14.04, p< 0.0001, Tukey’s multiple comparisons test).
Figure 18. *In vivo* BACE1 knockdown in the brainstem and cervical section of the spinal cord of mice, using worm- and rod-like, and spherical micellar nanoparticles. All infusions were performed in the right (ipsilateral) lateral ventricle at a dose of 16 µg of siRNA/day delivered using nanoparticles with the same dosing regimen as shown in Fig. 3b (n = 4 for infusion of worm-like, rod-like, and spherically shaped nanoparticles, where as scrambled and naked siRNA infusions were performed in duplicate). Protein blot analysis of BACE1 levels (a) in the brainstem and cervical section of the spinal cord after delivery of sequence BACE33 using worm-like (W), spherical (S) and rod-like (R) micellar nanoparticles. KO: BACE1 knockout, Co: vehicle infusion, Sc: Scrambled siRNA complexed with LPEI<sub>17k</sub>-g-0.8%PEG<sub>10k</sub>, N: naked siRNA sequence BACE33. (b,c) Quantification of BACE1 levels from protein blot analysis in the: (b) brainstem (Mean ± SEM, ANOVA, F = 24.83, p < 0.0001, Tukey’s multiple comparisons test), (c) cervical section of spinal cord (Mean ± SEM, ANOVA, F = 4.762, p < 0.05, Tukey’s multiple comparisons test).
Figure 19. *In vivo* BACE1 knockdown in the thoracic and lumbar sections of the spinal cord of mice, using worm- and rod-like, and spherical micellar nanoparticles. All infusions were performed in the right (ipsilateral) lateral ventricle at a dose of 16 µg of siRNA/day delivered using nanoparticles with the same dosing regimen as shown in Fig. 3b (n = 4 for infusion of worm-like, rod-like, and spherically shaped nanoparticles, where as scrambled and naked siRNA infusions were performed in duplicate). Protein blot analysis of BACE1 levels (a) in the thoracic and lumbar sections of the spinal cord after delivery of sequence BACE33 using worm-like (W), spherical (S) and rod-like (R) micellar nanoparticles. KO: BACE1 knockout, Co: vehicle infusion, Sc: Scrambled siRNA complexed with LPEI_{17k-g-0.8%PEG_{10k}}, N: naked siRNA sequence BACE33. (b,c) Quantification of BACE1 levels from protein blot analysis in the: (b) thoracic section of spinal cord (Mean ± SEM, ANOVA, F = 5.686, p < 0.05, Tukey’s multiple comparisons test), and (c) lumbar section of spinal cord (Mean ± SEM, ANOVA, F = 34.35, p < 0.0001, Tukey’s multiple comparisons test).
References


Appendix 1 *In vivo* Brain Microdialysis
Introduction

The extracellular environment of the brain is rich in information capable of revealing the underlying mechanisms of normal and abnormal function (Anderzhanova and Wotjak 2013). Microdialysis is a technique that lends itself attractively to the idea of providing one a “window” into the brain of awake and freely moving animals (Castellano, Kim et al. 2011). There is much that we can learn by keeping animals in their natural environment and studying the underlying processes in the brain. This method provides valuable information regarding a profile of a variety of substances found in the extracellular environment in an in vivo neurochemical context (Chefer, Thompson et al. 2009).

The method involves the use of a probe made up of a semipermeable membrane that separates the media being flushed through the probe and the brain tissue (Cirrito, May et al. 2003). The dialysate obtained from the media passing through the probe contain molecules that diffuse from the extracellular environment of the cell. The mass transfer is driven by a concentration gradient that exists across the semipermeable membrane (Morrison, Bungay et al. 1991). The size of the molecules being collected can be selectively controlled by choosing a probe with an appropriate molecular weight cut off. This technique is invasive and limited to a localized area of the brain (Chefer, Thompson et al. 2009, Anderzhanova and Wotjak 2013). But, considering that the extracellular content in any given region would be in equilibrium with the global pool, one can measure neurochemical responses in a timely manner. But, if large portions of the brain need to be studied in isolation, this technique will have its limitations. Additionally, the information we obtain from this method is obtain over the time frame of minutes to hours. One must be careful in the design of experiments prior to implementing this technique. If the goal of the experiment is to measure short term responses to stimuli one may have to turn to voltammetric techniques (Wightman and Robinson 2002, Robinson, Hermans et al. 2008).

Through in vivo microdialysis one can expect to be able to measure outcomes such as drug concentration in the brain, presence of neurotransmitters, peptides, hormones and metabolic
species in the brain. The maximum window over which this information can be collected is over a period of 96 hours. Following which the glial response and accumulation at the site of the semipermeable membrane would lead to a disruption in the equilibrium exchange of species in the tissue and perfused media (Cirrito, May et al. 2003). Interestingly this method can also be used to introduce very specific molecules to local area in the brain by means of reverse dialysis (Herrera-Marschitz, Goiny et al. 1997). Since the samples collected in the dialysate form are uncontaminated, they are readily amenable to downstream high throughput techniques (Guihen and O'Connor 2009). The samples measured by this technique provide real time data of the concentration of the species in brain tissue thus providing direct evidence relevant in the context of pharmacodynamic information.

As mentioned before, the processing of APP by BACE1 and γ-secretase leads to the formation of Aβ species that are released into extracellular space. While the manifestation of Aβ plaques is the hallmark of the disease in the late stages of progression, soluble Aβ species are released into the interstitial fluid bathing the CNS (Hong, Quintero-Monzon et al. 2011). In this context microdialysis has been judiciously employed to study the Aβ dynamics in the brain. As one would expect, the technique has lent itself towards being able to monitor Aβ level changes in response to a host of drug testing strategies (Chang, Huang et al. 2011). It provides valuable information in context of dose response in the form of Aβ decrease but also provides information how long the effect of the drug lasts in the form of Aβ levels reaching steady state levels once again. It is important to recognize an important consideration in the context of designing an experiment to measure Aβ levels. Prior to measuring any change in Aβ levels one must establish a stable baseline of Aβ levels prior to introducing any perturbation. This takes about 12 hours after implantation of the probe. The Aβ recovery in the dialysate is inversely related to the flow rate of perfused media (Anderzhanova and Wotjak 2013). Once a particular flow rate has been set, it must be kept constant for the duration of the measurement timeframe so as to not disturb the dynamics of Aβ exchange across the semipermeable membrane. Studies have increased our
understanding of Aβ levels under steady conditions and responsiveness to stimuli, for example the sleep wake cycles and activity dependent Aβ level changes (Kang, Lim et al. 2009). Additionally, by choosing an appropriate microdialysis probe reports have detailed the measurement of the protein tau in the CNS and their sensitivity to antisense therapeutics (Yamada, Cirrito et al. 2011).

At the present time an understanding of the pharmacodynamics of Aβ reduction in the context of nanoparticle delivery over the time span of delivery is challenging. Studies have had to employ the traditional approach of tissue harvesting techniques in order to gain a better understanding of the efficacy of the therapeutic approach being tested (in the context of nanoparticle delivery). Here we detail our efforts to bring together a powerful neurochemistry technique and ask very specific questions: how does Aβ level change in response to the infusion of nanoparticles in the brain? Does the knockdown of BACE1 at the levels that we have seen affect Aβ steady state levels? What are the key conclusions we make using this approach that might be useful for future studies?
Material and Methods

Setup

The goal of this effort is to put in place a setup to be able to use microdialysis to sample the interstitial fluid of the mouse brain. This setup can also be used for the infusion of material into the mouse brain as detailed in the previous sections. Due to the overlap between the two methods simple modifications at the final stages of setup can be made to switch from one experiment to the other.

The first step involves setting up the connection between a 1 ml syringe, with a 23G blunt needle, to the FEP-tubing (SciPro, Sanborn, NY). Fig 1. depicts the first two components that will be connected. After soaking the connector (SciPro) in ethanol based on the manufacturer’s recommendation it is placed on the needle such that the needle occupies the half-way point of the connector (Fig.2). The FEP-tubing is gently inserted from the other end of the connector such that it occupies the other half of the connector (Fig.3). The connection is allowed to dry at room temperature conditions for an hour so that the pink connector shrinks and makes a tight connection between the two components. It is important to note that up this point no solution is present in the syringe or in the tubing. When beginning to work with a variety of solutions that we were perfusing through the lines it was important to ensure that no air bubbles were introduced. To ensure this, a couple of precautions were taken; the needle cap was first filled with the solution to the point that we observed a small bubble of fluid (Fig. 4). The syringe was now filled with solution to the point that a bubble is observed at the tip of it as well (Fig 5.). At that point the syringe and needle were attached (Fig.6). By ensuring the contact between the excess liquid in the two parts we were able to minimize the possibility of bubble formation while making the connection. Using a slow infusion pump the solution is injected through the tube at 1.0-2.0 µl/min. At the other end of the tube liquid is allowed to flow out (Fig.7). The cannula from the infusion apparatus is now connected to the tube in a similar manner as stated above (Fig.8). Note that clear connectors (BASi) were used this time to ensure a tight connection. All connections for
future steps should be made with clear connectors. The pink connectors have been shown in the figures for a better visual representation.

Prior to using the microdialysis probe (BASi), it needs to be equilibrated with solution that will be flowing through it. We placed the probe with an appropriate clamp such that the semipermeable membrane is in contact with a solution in a 1 ml eppendorf tube (Fig. 9). The equilibration time was at least 10 minutes. The free end of the tube (Fig.7) was then connected with the dialysis probe. At first the pump was stopped once liquid began to flow out of the tube. The connector (following alcohol treatment) was then attached to the free end of the tube (Fig.10). The pump was starting once again till liquid began to flow out of the connector. At that point the connector was connected to the yellow, inlet, port of the dialysis probe (Fig. 11). The pump is switched off for about 10 minutes so as to allow the connection to tighten. The pump is started up once again. It was important to ensure that the integrity of the dialysis probe was not compromised at any point. To this end, after the pump was started and solution is flowing through, the probe was removed from the equilibrating buffer (1 ml tube) and checked to see if there was any solution leaking out (Fig.12). Once the probe membrane has been wetted care must be taken to keep it in an appropriate environment so as to prevent drying. After verifying that there was no leak through the semipermeable membrane, we observed that liquid flows out of the green, outlet, port of the connector. This time a connector is attached to the green port first followed by connecting a separate tube at that junction (Fig.13). The free end of this tube will contain the dialysate (solution with material exchange with tissue in the brain). The solutions eluting at a constant flow rate can be collected at a variety of time points and analyzed for their molecular content. For this particular study we focused on analyzing the eluted samples using ELISA to assess $\text{A}\beta_{40}$ content based on the manufacturer’s recommended protocol (Life Technologies).
Animal Surgery

Mice were placed in a stereotaxic apparatus so as to immobilize the skull prior to any implantation of the probe/cannula (Fig.14). Prior to placement of the animals on the stereotaxic apparatus, they anesthetized using 5% isoflurane. Once the animals were placed on the apparatus, a nose cone was used to regulate the flow of isoflurane, and maintained at approximately 1%. Following anesthetization, the hair above the skull of mice was removed to expose the scalp (Fig. 15). The skin is thoroughly cleaned and an incision was made along the midline to expose the skull (Fig.16). A hole was drilled through the skull, above the right lateral ventricle (bregma – 0.5mm,1.0mm lateral) (Fig.17). After drilling, bone fragments were cleaned away. Alzet (brain infusion kit# 3, Cupertino, CA) apparatus, as per manufacturer’s specifications, was used to place a cannula at a depth of 2.2mm (Fig.18). The cannula was cemented using dental cement. A long tube (FEP-tubing, SCIPRO) was used to connect the end of the cannula above the skull to a slow infusion pump (Stoelting Co. Wood Dale, IL). The tube connecting the slow infusion pump and the cannula was sufficiently long so as to allow free head and neck movement of the mice. The animal was then placed in a special enclosure – Raturn Microdialysis Stand-Alone System (with free access to food and water) where the tube going to the slow infusion microdialysis pump can be secured and the process of infusing the therapeutic agent was begun (0.1ul/min – during the infusion phase). At any given point there would be only one mouse present in the Raturn Microdialysis Stand-Alone System undergoing infusion (BASi, West Lafayette, IN). We used a slow infusion pump considering that we need the flexibility of have a system that would be able to deliver agents from a period varying from 2 days up to 7 days with the ability to stop infusions, as per our protocol. Importantly it allowed us to deliver a specific volume of therapeutic to the targeted area in the brain and allowed us to monitor the effect in the live animal which would mimic a clinical setting where the therapeutic can be potentially used. In this report the right hemisphere is referred to as the ipsilateral side of the brain and the left hemisphere is
referred to as the contralateral side of the brain (with reference to the side of infusion in the brain). The protocol detailed here is similar to the one stated in the previous section.

For microdialysis: Following anesthetization the hair above the skull of the mouse was shaved so as to expose the skin. Using a scalpel an incision was made along the midline to expose the skull. Due to the incision there will be minor bleeding. Sterilized cotton swabs were used to clean the skull with wet with aCSF (artificial cerebrospinal fluid). For microdialysis probe implantation, two 0.7mm holes were drilled through the skull. One hole above the left hippocampus (bregma – 3.1mm, 2.4mm lateral). A similar hole can be drilled over the right hippocampus if desired. A second hole was drilled into the skull opposite to the hemisphere of the first hole. A bone screw was inserted in the skull surface for the purposes of anchoring our apparatus on the mouse brain. A sterile guide cannula (BASi) was inserted into the brain region of interest. It was then fixed into place using dental cement. Once the cement solidified the animal was removed from the stereotaxic apparatus and the sites of the initial incision were closed using surgical glue. After 24 hours, microdialysis probes (35-kilodalton molecular weight cut-off membrane, BASi) were inserted through the guide cannula while the animal was briefly anesthetized in the Raturn chamber. The tubes exiting the microdialysis probe (Fig. 13) were connected to a slow infusion pump and a collector for the dialysate respectively. The slow infusion pump would ensure continuous passing of the dialysis sample via the probe to a fraction collector. For the case of merging infusion with microdialysis with infusion, the infusion probes were typically placed on the right side of the brain. This meant that the microdialysis probe was placed on the left side of the brain. Critical care must be taken to ensure that the components are suitably spaced. The baseline for Aβ levels were first established with the presence of the cannula (but no infusion into the lateral ventricles). Following the initial collection of samples at a variety of time points the infusion process was started and then stopped at a later time point. Once again eluted samples were analyzed using ELISA to assess Aβ40 content based on the manufacturer’s recommended protocol (Life Technologies).
Results

In order to assess that we can measure Aβ levels in a stable and reproducible manner we first used only a probe to assess protein level in an APP/PS1 transgenic mouse and a litter mate control (non-transgenic mouse). We note here that we were able to establish a stable baseline of measured Aβ₄₀ (Aβ) levels over a time span of eight hours in the absence of any perturbation (Fig.19). Samples were collected every two hours. This verification of experimental method in our hands was very important so as to continue future studies, since every conclusion of a perturbation is based on the deviation from baseline values. To verify the sensitivity of the ELISA assay to measure Aβ levels from the dialysate captured by the microdialysis method, we analyzed fractions collected from the non-transgenic littermate and noted that the signal was at background levels (Fig.20).

Having established the sensitivity of the assay we then proceeded to measure Aβ levels from the hippocampus (left hemisphere) while infusing, either vehicle (5%glucose) or siRNA loaded nanoparticles, in the right lateral ventricle of mice. It is important to note here that throughout the dialysis/infusion procedure the animals are freely moving and have continuous access to food and water in the Raturn apparatus. We first measured Aβ level changes in response to the infusion of vehicle in the lateral ventricles of the mice (Fig.21). After establishing a baseline level of Aβ prior to infusion we observe that the levels increase over the time span of infusion and continue to be shifted higher (with fluctuations close to initial baseline levels). This reflects in a change in Aβ levels purely in response to the infusion process of vehicle. Fig. 22 depicts the changes in Aβ levels in response to infusion of nanoparticles over a time span of two days (16 µg of siRNA per day). The rapid drop in Aβ levels noted after the start of the infusion is due to the sudden infusion of a slightly larger volume (than programmed in the pump). The drop in Aβ levels exquisitely reflects the sudden infusion of a larger than expected volume in the brain. Following which the Aβ levels continue to fluctuate in a manner similar to the infusion of just vehicle. Importantly, no decrease in Aβ was noted as compared to the initial established baseline value.
We then reasoned that the dose of siRNA targeting BACE1, packaged in nanoparticles, was spread over a period of two days and hence not be able to affect the steady state levels of Aβ. We then decided to infuse 32 µg of siRNA, encapsulated in nanoparticles over a time span of eight hours to observe if we might be able to measure dynamic changes in Aβ below the baseline level (Fig.23). Additionally we anticipated that any discernible changes in Aβ would require a significant amount of time to be observed (contrary to what one would expect with the use of small molecule drugs). We decided to monitor the changes in Aβ levels for the following 48 hours after the infusion had been stopped. Once again we observed that Aβ levels did not drop beyond the initial baseline levels. We continue to see a fluctuating pattern of Aβ measurement upon commencement of the infusion process.

From the knockdown studies of BACE1 we realized that we observed a discernible change in BACE1 levels after infusion of nanoparticles over a four day time span following the staggered infusion protocol. We then decided to increase the dose to 64 µg of siRNA packaged in nanoparticle form, and infuse it over a very short time span of three and half hours (Fig.24). We once again monitored Aβ levels past the infusion step of the process and noted that the Aβ levels did not drop beyond the initial baseline levels established prior to the infusion of nanoparticles in the right lateral ventricle.
Discussion

Microdialysis has been successfully employed to show the effect of the use of drugs, targeting BACE1 and γ-secretase, on Aβ levels in a variety of mouse models (Elvang, Volbracht et al. 2009, Ulrich, Burchett et al. 2013, Fitz, Castranio et al. 2014). Since we commenced this work with the goal of being able to modulate the players implicated in AD, this technique provides an ideal window to be able to study the efficacy of our designed therapeutic approach. We have learned thus far that the use of shaped nanoparticles can lead to a knockdown of around 40% of BACE1 levels. These reductions in BACE1 were observed at the end of 7 day staggered infusion protocol.

We have been able to successfully replicate the microdialysis setup and experimental methods (generously shared with us by the Holtzman Lab). This is evidenced from the comparison between the Aβ levels between the transgenic and the non-transgenic animal. The non-transgenic sampling replicates a pseudo BACE1 inhibition that shows that the measured amount of Aβ is at background levels. All of the infusion studies that we have detailed by varying the dosing regimen and extending the time period of measurement of Aβ levels after stoppage of infusion did not yield a significant decrease in Aβ. This can be explained from the fact that it has been shown from genetic studies that a 50% reduction in BACE1 levels leads to about a 20% reduction in the activity of the enzyme (Yan and Vassar 2014). In addition it is known that BACE1 activity is upregulated in the presence of injury in the brain (Guglielmotto, Aragno et al. 2009), which in our experimental setup is mimicked in the form of two probes being implanted in the brain of the mice. These attributes make it extremely challenging to be able measure changes in BACE1 activity or levels at the present knockdown levels that we have achieved. Indeed current pharmacological candidates that inhibit BACE1 by close to 80% have shown a discernible downstream effect in terms of Aβ reduction (Forman, MS et al. 2013).

While the reductions in Aβ were not discernible for our studies, this method of coupling nanoparticle infusion with microdialysis can be suitably applied for studying perturbations in
other disease pathways (for example tauopathies). Interestingly one might be able to envision the use of this method to be able to observe transient changes in biomarkers in a variety of disease models when testing/discovering new therapeutic approaches. More importantly the next generation of nanoparticles that can be built on this platform with the use of targeting ligands would be better able increase the delivery efficiency of the nanoparticles whose efficacy can then be captured in real time by monitoring the neurochemistry of the brain.
Figure 1. Making the initial connection. Pink connector is to be used as an attachment between the tubing and 23G blunt needle.
Figure 2. Setting up the connection between the needle and the connector. After soaking the connector in ethanol (as per the manufacturer’s recommendation) the connector is placed in such a way that half of it is occupied by the needle.
Figure 3. Setting up the connection. After soaking the connector in ethanol (as per the manufacturer’s recommendation) the connector is placed in such a way that half of it is occupied by the needle and the other half by the tubing.
Figure 4. **Ensuring no air bubbles enter the system.** The needle holder is filled with the solution of choice to the point that it protrudes and forms a bubble.
Figure 5. Ensuring no air bubbles enter the system. The syringe is filled with the solution of choice to the point that it protrudes and forms a bubble.
Figure 6. Making the connection. The filled syringe with solution is attached with the needle at this point. This ensures that no air bubbles enter the system.
Figure 7. Flushing the tubing lines with solution. Ensuring that the solution flows out of the tube for future connections is crucial.
Figure 8. Connecting tubing to cannula. Clear connector used for this connection. Same principle of making connections is used here.
Figure 9. Setting up the dialysis probe. The dialysis probe is equilibrated in an artificial CSF solution for 10 minutes.
Figure 10. Ensuring flush connections. Solution if flushed through the tubing and the connector.
Figure 11. Connecting tubing to the probe. The connector is placed on the yellow inlet. Pink connector is shown here for visual purposes. Clear connectors are to be used at this point.
Figure 12. Probe inspection. Ensuring that no solution leaks from the semi-permeable membrane is important.
Figure 13. Making the final connection. Connection is made between the tubing and the green outlet. Pink connector is shown here for visual purposes. Clear connectors are to be used at this point.
Figure 14. Mouse placed in stereotaxic apparatus. Holders are placed to ensure immobility of the head after the animal has been anesthetized and continues to remain under anesthesia.
Figure 15. Hair removal. Hair removal cream applied for removal of hair.
Figure 16. Exposing the skull. Mid-line incision is made to expose the skull.
Figure 17. Selecting a point for drilling. Coordinates based on brain atlas used to drill a hole carefully through the skull.
Figure 18. Cannula placement. Cannula is carefully lowered to selected depth. In a similar manner the microdialysis probe can also be placed in the brain.
Figure 19. Aβ baseline levels in a non-transgenic APP/PS1 animal. We note here at Aβ levels are stable and can thus establish a baseline for measurement of any perturbation.
**Figure 20. Aβ levels in a non-transgenic animal.** We note here at Aβ levels are at background signal levels. Thus, establishing the sensitivity of the assay.
Figure 21. Aβ level changes in response to infusion of vehicle over two days. We note here at Aβ levels baseline shift higher when infusing vehicle over a two day time span.
Figure 22. Aβ level changes in response to infusion of nanoparticles over two days. We note here at Aβ levels remain unaltered when infusing nanoparticles targeting BACE1 over a two day time span.
Figure 23. Aβ level changes in response to bolus infusion of nanoparticles over 9 hours. We note here that Aβ levels remain unaltered when infusing nanoparticles targeting BACE1 with this dosing regimen.
Figure 24. Aβ level changes in response to bolus infusion of nanoparticles over 4 hours. We note here at Aβ levels remain unaltered when infusing nanoparticles targeting BACE1 with this dosing regimen.
References


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Curriculum Vitae

Rishab R. Shyam

24th March, 2015

Education
Johns Hopkins University, Baltimore - Ph.D. Candidate in Biomedical Engineering
(Expected May 2015)
Thesis: Nanoparticle Based siRNA Delivery to the Central Nervous System: A Potential Therapeutic Approach

Purdue University, West Lafayette - B.S. in Chemical Engineering with Minors in Economics and Chemistry (May 2006, Distinction and Honors, GPA: 3.94/4.0)
Thesis: Understanding and Postulating Reaction Sequences for Aromatization of Propane on HZSM-5 catalyst

Other Professional Experience

Johns Hopkins University Entrepreneurship Program (Aug ’14)
- Focused on commercializing an idea for RNAi therapeutic development based on provisional patent from graduate work
- Lead a team of 5 graduate students over a 3 month effort towards building a road map of taking an idea from the bench to the clinic
- One of 4 teams selected from 22 other teams to make a live pitch to seven investor/entrepreneurs
- Assigned a mentor from the Maryland Innovation Initiative to build on approach for early stage of funding

Instructor: BME Intersession Course, EN.580.108.13, Neurological Diseases to Nanoparticles (Jan ’13)
- Designed for credit course involving lectures coupled with labs, for a class of 24 Hopkins under-graduate students, emphasizing the interdisciplinary nature of scientific research
- Focused on getting students to appreciate fundamental scientific concepts with real world examples
- Dealt with the nature of experimental planning from hypothesis to execution
- Created a spring board for students to continue their exploration in science through undergraduate research fellowships and exposing to them to research work performed at Hopkins

Awards and Honors
- Awarded American Society of Gene & Cell Therapy Meritorious Research Travel Award for oral presentation on ‘Delivery of Therapeutics to the Central Nervous System Mediated by Shape-Controlled Nanoparticles’ (May ’14)
- Dean’s List and Semester Honors: Purdue University (Jan ’02 – May ’06)
- Department of Chemical Engineering Fund Scholarship (Jan ’05)
Peer-Reviewed Publications

Intellectual Property

Oral Presentations
1) **Shyam R**, Ren Y, Lee J, Braunstein KE, Mao HQ, Wong PC. Delivery of Therapeutics to the Central Nervous System Mediated by Shape-Controlled Nanoparticles. American Society for Gene and Cell Therapy, Annual Meeting in May 2014.
2) **Shyam R**, Ren Y, Braunstein KE, Mao HQ, Wong PC. Nanoparticle Mediated siRNA delivery to the Central Nervous System. Division of Neuropathology Seminar, Johns Hopkins University, Jan 2013.

Leadership/Community Involvement
Invited Speaker at Ignite Hopkins Forum: Talk about ‘The Art of Sprinting without Sprinting’ (Mar ’14)
- Shared ideas about mentorship and lessons learnt from volunteering experiences
Chairperson, Fly-out Committee, Experimental Aviation Association, Westminster, MD (Jan ’14-Present)
- Licensed Private Pilot for a single engine airplane, since 2012, and have logged 235 hours of flying time
- Evaluated risk parameters such as weather conditions and flight safety
Co-organizer of annual Biomedical Engineering fund raising events for United Way (Dec ’12 & Dec ’11)
- Reached out to approximately 100 students and faculty in an effort to raise fund for charitable causes
Member of Fund Raising Committee, Incentive Mentoring Program (Jan ’11-Dec ’11)
- Grant writing focused on highlighting organizational achievements and core ideas to donor organizations
- Developed strategies for effective communication of goals to donors via qualitative and quantitative means