Honors Thesis Proposal

For

Alpha-Synuclein: Insight to the Hallmark of Parkinson's Disease as a Potential Biomarker for Aptamer-Based Theranostics

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Specific Aims

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide, caused by intracellular inclusions of aggregated alpha-Synuclein (α-SYN), which result in dopaminergic neuronal death. This pathological cascade imparts severe neurological motor, behavior, and sensory deficits. Research has been aimed at utilizing aggregated α-SYN deposits (Lewy bodies) as biomarkers for diagnostics and therapeutics. Much is to be improved upon PD diagnostics—qualitative assessments contingent upon physicians' clinical experience—and therapeutics—that paradoxically induce PD-like symptoms with prolonged regimes. This project serves to explore α-SYN as a potential biomarker for a biologically compatible "theranostic" that provides simultaneous diagnosis and therapy utilizing antibody-like nucleic acid aptamer and the anti-fibrillogenic peptide: Protein Disulfide Isomerase (PDI). The specific aims for this project are as follows, establishing:

Aim 1: A Split-Spinach Aptamer for Detection of oligomeric α-SYN

A previously reported anti-oligomeric α-SYN aptamer will be designed for novel split-spinach analysis to detect oligomeric α-SYN in vitro. A series of negative controls will be examined to ensure the specificity of the aptamer. A calibration curve will be established to determine the limit of detection for the construct and further methods of signal amplification will be explored

Aim 2: The Ameliorative Effects of PDI in Live Cells Expressing Aggregated α-SYN

A PDI over-expression vector will be cloned and co-transfected with a Gaussian luciferase α-SYN complementation system to comparatively analyze PDI's effect on preventing and/or reversing α-SYN oligomerization.

Background and Significance

Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disease with incidents making it the second most common neurodegenerative disease after Alzheimer's disease [1]. The disease phenotype is characterized by any combination of four diagnostic features: resting tremor, bradykinesia, muscular rigidity and postural instability due to dopaminergic deficit in the nigrostriatal region of the midbrain[2]. Current epidemiological data reveals that as of 2005, more than 4 million were diagnosed world-wide, with a 0.3% prevalence in the general population and a male: female ratio of 3:2[1]. The general cause of PD is unknown and largely debated as both idiopathic and genetic forms of the disease are prevalent in the clinical setting. Diagnosis and treatment of PD are limited, likely due to the inherent difficulty in delineating several near-identical symptom profiles found in other neurodegenerative diseases[3]. Much attention for targeted diagnostics and therapies has been turned to a key molecular hallmark of PD, alpha-Synuclein (a-SYN), which exists as aggregated intraneuronal inclusions in the PD brain [4].

Clinical Diagnosis of PD

As previously mentioned, the cause of PD is elusive, with early and/or prophylactic diagnoses remaining a challenge during pathogenesis[5]. Genetic screening is available, however is only applicable in a limited number of cases [6]. It has been noted that environmental, genetic, and epigenetic influences can be responsible for an individual's propensity to PD at varying ages in life. Typically, the disease occurs infrequently under 40 years of age, coined *familial* PD, however is most prevalent in patients >65 years of age,

clinically denoted as *idiopathic* PD. In the United States alone, there exists an excess of 500,000 patients, with 50,000 new patients diagnosed yearly[7].

Four aforementioned motor characteristics denote a symptomatic patient in the clinical setting.

1) Bradykinesia is defined as the loss of speed in performing repetitive movements of extremities, and is easily confused with simple slowness encountered during advanced age. A subset of bradykinesia can include any combination of *hypomimia, hypophonia, micrographia, and dysphagia* (decreased or absent facial expression, diminutive vocal projection, diminutive handwriting, and difficulty swallowing, respectively)[2].
2) Resting tremor, otherwise known as *parkinsonian* tremor, is the presentation of subtle, rhythmic movements when the body is not engaged physically or mentally. The most notorious clinical example of resting tremor is known as "pill-rolling" in which the patient involuntarily rubs the flat of their pollex and index finger together in minute circular patterns[8].
3) Rigidity is classified as abnormal increase in muscle tone during movement of the *both* flexor and extensor muscular analogs, in either the neck or extremities. This incongruity amongst muscle groups leads the patient to experience "cog-wheel" movements where movement in one muscle group will trigger simultaneous movement of another group that otherwise would not be integrated[9].
4) Gait Impairment is of the final and most identifiable symptom of a PD patient. Often, the patient will exhibit "anterior truncal flexion" whereby the upper body

leans inward, less than 20 degrees relative to normal. This is accompanied with shuffling footsteps where the feet are devoid of dorsal flexion or plantar flexion during walking. To conclude gait impairment, a physician will employ a "pull-test" to gage the individual's ability to maintain a stable posture or observe "chasing" of their center of gravity after a mildly destabilizing stimulus [10].

Current Clinical Treatment of PD

To date, several interventions of PD, both pharmacological and surgical, exist to a semi-efficient degree, making therapeutics more advanced in terms of clinical competency when compared to diagnostics. However, a substantial amount of adverse effects arise from both, regardless of the strict guidelines physicians follow to ensure proper treatment. From the pharmacological perspective, subclasses include: Levodopa, Catechol-o-methyl-transferase (COMT) inhibitors, Dopamine agonists, and non-dopaminergic drugs as well as neurosurgical approaches[11].

Levodopa (L-DOPA) or L-3, 4-dihydroxy-phenylalanine, is a chemical analog of dopamine. Because dopamine has a terminal amine conserved from its amino-acid precursor, tyrosine, its highly polarized dipole-moment inhibits the ability to penetrate the blood-brain-barrier (BBB) and as such cannot be used as a drug with the aim of compensating for the decline of intrastriatal dopamine[12]. Levodopa, however, maintains its amino-acyl backbone and is able to permeate the BBB where upon interaction with DOPA decarboxylase, reduces the carboxy-termini to a secondary amine yielding the production of intracerebral dopamine. Despite it being the most popular, as well as potent pharmacologic available for PD, L-DOPA in itself can induce motor pathologies such as dyskinesias, and therefore L-DOPA is in a constant state of debate regarding its clinical

efficacy[13]. At times, a peripheral DOPA-decarboxylase inhibitor, carbidopa, enhances the drug's potential by limiting the activity of dopa-decarboxylase in regions of the body other than the brain. It has been reported that patients prescribed L-DOPA will typically experience motor deficits after five years on the regiment which leads some physicians to withhold L-DOPA therapies until deemed fit so as to postpone the onset of the adverse side effects. It has also been noted that early-onset PD, as well as genetic forms such PARK2 and PARK8 are especially susceptible to the development of L-DOPA related motor deficits [14]. Countermeasures for the amelioration or prophylaxis of L-DOPA dyskinesias include titration of the dosage, additional medications, or even surgical intervention.

Alpha-Synuclein

As previously mentioned, PD is likely the result of imposed dopaminergic cytotoxicity due to the presence of intracytoplasmic inclusions of α-SYN[15]. α-SYN, otherwise monomeric in the non-pathological state, forms insoluble higher-order aggregates and likely compromises the integrity of the plasma membrane, leading to progressive neurodegeneration[16]. The mechanism by which α-SYN undergoes insult and subsequent aggregation has therefore been a great focal point for PD etiology. Two prevailing proposals for aggregation include 1) malfunctions in the ubiquitin proteasome system specificity due to intratress induced by free radicals and reactive oxygen species (ROS).

1) UPS is the primary biochemical mechanism by which cells on a systemic level reduce, reutilize, and degrade both normal as well as abnormal intracellular proteins through a two-step signaling cascade. A protein targeted for degradation will be acted upon by several ubiquitin-conjugating enzymes, through which interaction with the 26S proteasome will occur [17]. Interaction

and recognition of the ubiquitin tags induces ATP-dependent proteolysis within the 20S core cylindrical unit, releasing small peptide fragments from the 19S cap to be further degraded in the cytosol via transaminases and transpeptidases[18]. The cascade is completed upon the release of reusable ubiquitin components. Failure or down-regulation of the UPS can cause an intracellular congestion of proteins intended for degradation, and is implicit in the beginnings of neurodegeneration. Post-mortem analysis of the substantia Nigra pars compacta Lewy Bodies reveals the presence of ubiquitin polymers as well as fragments of the 26S proteasome. Biochemical analysis reveals proteins tagged for degradation bear monomeric rather than polymeric ubiquitin tags, suggesting that insufficient ubiquitination contributes to an inefficacious degradation pathway and subsequent PD pathogenesis[19].

2) The presence of oxidants has been well established as a method by which protein insult occurs. In vitro, it has been demonstrated that exposure of α-SYN to hydrogen peroxide and iron facilitates the aggregation of higher-ordered structures and formation of cytotoxic intermediates [20]. In a process coined the Fenton effect, cultured cells treated with traces of ferrous chloride demonstrate a heightened aggregation potential evident through Western blotting, immunocytochemistry, and cytotoxicity assays. Similar results are seen through use of hydrogen peroxide with the added effect of ROS-mediated mitochondrial dysfunction and cell death[21]. Studies involving mutated α-SYN (A53T, S42Y, and A30P) illustrate a hyper-susceptible aggregation response to oxidative species. The proposed mechanism by which oxidation-induced aggregation in PD

proceeds involves the chemical nature of dopamine. An inherent free-radical generator, as well as principal neurotransmitter in the SN, dopamine's reactive chemical nature is compounded by free radical-generating iron which accumulates in neurons with advancing age, and substantiates a likely hypothesis as a hallmark pathogenic mechanism[22]. Advances in studying this process in vivo are being made to avoid the commonly used and highly potent environmental toxins MPTP, Paraquat, and Rotenone, which induce neuronal death faster than LB formation[23].

While a definitive physiological role and mode of pathogenesis remain speculative and surmised from the biochemical properties of α-SYN, the distinction between the monomeric and aggregated ultrastructures potentiates a clinically applicable biomarker for LB-related synucleinopathies such as PD.

Aptamers

Aptamers are a class of functional nucleic-acid compounds receiving a significant amount of scientific exploration at the academic and industrial level with wide-scale applications targeted toward the clinical setting. Aptamers are composed of synthetic ssRNA or ssDNA, which, due to intra-nucleic acid hydrogen bonding, achieve higher-ordered secondary and tertiary structures that confer a functional "lock-and-key" property to specific substrates in an antibody-like fashion[24]. A functional aptamer ranges in size from ~20-100nt, comparatively 20-25 fold smaller than the monoclonal antibody analogs, which allows for an increased penetrable ability across a variety of biological membranes and barriers such as the glomerular-Bowman Capsule interface [25]. Furthermore, these moieties are nucleic-acid polymers, not amino acid constituents, which starkly reduces immunogenic

response in vivo. Furthermore, unlike antibodies, aptamers can distinguish between structural variants of proteins such as monomers and oligomers, whole-cells, or tissues; qualities that make aptamers substantial technology for use in clinical applications such as diagnostics, therapeutics, or synergistic "theranostics"[26].

Aptamer Synthesis

Aptamer synthesis was well established in 1990; however translational applications were not possible until recently when intellectual property patents on the mode of synthesis expired, leading to a surge of aptamer-based research. Biochemists Tuerk and Gold coined the process *Systematic Evolution of Ligands by Exponential enrichment* (SELEX) as they sought to select a library of RNA ligands that interacted with T4 DNA Polymerase[27]. This iterative process begins with a randomized library of oligonucleotides, or "oligos" linked to a ubiquitous priming region. The oligos are heated in folding buffer that contains 1mM $MgCl_2$ to 95°C to denature the oligos and disrupt randomized intramolecular binding. The solution is then allowed to cool to room temperature at approximately 1°C per minute while the Mg^{2+} cofactor allows the oligo to self-anneal according to the most energetically favorable secondary or tertiary structure, creating a functional aptamer. The randomized aptamers are then incubated with target antigens as well as counter-selection compounds[28]. Those sequences that interact with counter-selection compounds are discarded while those that demonstrate binding to the target compound are amplified via PCR or RTPCR (for RNA moieties) with primers that anneal to the ubiquitous priming regions in the oligo library. This selection and amplification process is then repeated 10-20 times. Final selection will yield several aptamers with conserved sequences but variable dissociation constants as determined by surface-plasmon resonance. This process can also

be carried out using live cultured cells as a *de novo* process through which novel biomarkers can be established for specific cell types. Advantages of SELEX compared to animal-mediated antibody production include efficient production scaling, reduced production time, and a predictable chemical nature that allows for homogenous conjugation to fluorophores, quenchers, and drug cargo[26].

In-Vivo Applications

Aptamers intended for in vivo applications must be chemically modified to withstand dynamic biological conditions not otherwise encountered in in vitro assays. A drawback to aptamer chemistry is degradation of the uncapped 5' and 3' termini upon interaction with exonucleases. Furthermore, the phosphodiester backbone in pyrimidine residues can be targeted by ribonucleases leading to internal degradation and loss of functional conformation. However, through the use of modified nucleotides, the stability of aptamers for in vivo studies can be efficiently, and cost-effectively ameliorated. Inverted 2'-o-modified pyrimidine nucleotides such as 2'-amino pyrimidines and 2'-fluoro pyrimidines render endo/exonucleases catalytically inactive[29]. It is imperative that inverted bases as well as high molecular weight cargos be incorporated into the oligo library before SELEX as the modifications can alter dissociation constants or even specificity due to intramolecular interactions conferring altered free -energies and conformations[30].

Aptamers selected for a target molecule with a G-quadruplex structure, can be split into two half-sequences. Each half-sequence can then be covalently linked to one-half of a catalytic nucleic acid structure. When introduced to the target ligand, each half of the antigen-binding sequences interacts, bringing the catalytic segments in close proximity to each other. When the substrate, 3,5-difluoro-4-hydroxybenzylideneimidazolinone, (DFHBI)

is introduced to the solution, the now active catalytic region cleaves the substrate to produce a highly fluorescent signal detected via fluorimetry. Because this process occurs in solution and adheres to enzyme-kinetic laws, the level of fluorescence can be influenced by the concentration of DFHBI substrate as well as incubation time and temperature, potentially allowing for a highly sensitive means of detection, sometimes on the order of femtograms (1×10^{-15}). Although this method was originally intended for the detection of foreign nucleic acid from microbial pathogens, the use of split-systems for detecting protein and other biomolecules is currently under investigation[31].

Within the past five years, a considerable amount of study has been invested with the aim of surmounting the limitations to aptamer-based neurodegenerative diagnostics and therapeutics including:

1) *Biomarkers for neurodegenerative diseases are often higher ordered structures of peptides that are nevertheless found in monomeric states in non-pathological conditions and therefore are composed of identical chemical sequences that should be differentiable by the molecular probe*

Ikebukuro et. al has demonstrated the production of aptamers selected for oligomeric a-SYN only. Using the typical SELEX scheme, the aptamers were counter-selected against monomeric and fribrillar forms, so as to enable researchers to detect the presence of cytotoxic species before LB production and subsequent neurodegeneration occur. Biochemical analysis via TfT analysis suggests aptamers T-SO517 and T-SO606 produce detectable responses against immobilized a-SYN only when TfT intensity surpasses 1000 A.U. indicating the presence of higher-ordered species. Aptamer T-SO508, while showing the strongest reaction to oligomeric a-SYN, also detected AB due to the conserved beta-

edge conformation. However, because this type of motif is present in a wide variety of proteins (IgG, CRP, albumin), a sufficient negative selection is necessary to produce highly specific probes for use in physiological environments[28].

2) *The blood-brain-barrier (BBB) inhibits upwards of 80% of polar, charged molecules such as DNA*

The BBB is a major hurdle in diagnosing and treating neurodegenerative diseases. While several drugs and probes have shown promise at the neurological level, achieving their final destination in a non-invasive manner leaves room for improvement[32]. In 2014, Freskgard et. Al demonstrated increased brain penetration of a therapeutic monovalent antibody for AB plaques (mAb31) achieved through monovalent engagement of the Transferrin receptor (TfR). Engagement of this receptor at the BBB microvasculature allows for receptor-mediated transcytosis through the polarized endothelium, delivering cargo through the abluminal region into the cerebral parenchyma. Within 15 minutes, intravenous injection of the antibody chimera displayed marked penetration and plaque decoration. Approximately 4.5 months post-treatment of PS2APP amyloidosis mouse models suggested an increased therapeutic effect in the reduction of plaques 8-16μm^2 when compared to treatment without TfR engagement. This study has shed light on the significance of receptor engagement for non-invasive shuttling across the BBB, however antibody-based reactions of the TfR and parenchymal delivery can impose several disadvantages such as: intra-endothelial lysosomal sorting and degradation upon divalent interaction of the receptor, as well as an induced immunogenic reaction as a result of the large peptide-based IgG molecules[33]. Beltram et. Al, utilized a similar concept of TfR engagement as a means of CNS delivery, however utilizes anti-TfR aptamer ATTO 633 as

opposed to antibodies. Because aptamers display monovalent modes of binding, there is a reduced chance of lysosomal sorting at the BBB endothelium[34].

3) *Biomarkers for neurodegenerative diseases, such as a-SYN aggregates, are intracellular inclusions, therefore aptamers must be capable of freely accessing the plasmalemma*

Because a-SYN deposits are intracellular, another hurdle to engage the LB for diagnostic and/or therapeutic purposes is the ability and efficiency by which the molecular probes can diffuse through the neuronal plasma membranes without imparting their own degree of cellular damage after CNS access. Further metabolic differences between neurons and the polarized endothelium of the BBB necessitate dynamic functions of the molecular shuttle. In endothelium, or BBB interface, maintenance in the transport vesicle is imperative to be completely transcytosed into the BBB, however at the neuronal interface, vesicular release in the cytoplasm is required for interaction with the cytosolic target in a process dubbed endosomal release. Battaglia et. al illustrated engagement of Low Density Lipoprotein Related Protein 1 (LRP-1) receptor facilitates the aforementioned requirements. pH-sensitive polymersomes containing nucleic acids, coated with LRP-1 ligand Angiopep-2, triggers transcytosis at the BBB endothelium. Once in the parenchyma, these constructs are then able to engage in traditional endocytosis by the neurons without intracellular lysosomal sorting and circulate freely in the cytoplasm of the neurons. Advancements in aptamer- and nanotechnology have established several modes by which diagnostic or therapeutic aptamers can self-facilitate target engagement with minimally invasive and diminutive toxic effects in vivo[35].

Protein Disulfide Isomerase

Protein Disulfide Isomerase is a five-domain, 55kDa protein that exerts a chaperone-like quality in cells. It is constitutively produced in millimolar concentrations, however is predominantly localized in the endoplasmic reticulum. The enzyme possesses an oxidase activity to introduce disulfide bonds into proteins as well as an isomerase activity to induce intramolecular shifts[36]. This functionality has been explored as a means of isomerizing the intramolecular bonds by which proteins achieve higher-ordered, oligomeric structures. This enzyme evolved as a mediator to redistribute bonds as synthesis in the ER occurs, which is an error-prone process, until the correct protein structure is conferred[37]. Because several diseases are associated with abnormal protein aggregation including PD, Alzheimer's disease, and other amylodogenic metabolic disorders, much attention has been turned to the amelioration of such diseases by utilizing an intrinsic property of the native enzyme[38].

Research Design and Methods

Aim 1: A Split-Spinach Aptamer for Live Intracellular Detection of α-SYN

Aim 1.1: Screening Aptamer to Determine Specificity

Because aptamers can be influenced by temperature and pH, and are structure-specific rather than sequence-specific, the absence of cross-reactivity is imperative for clinical advancement[28]. As such, the aptamer will be screened sequentially against thrombin, bovine serum albumin, and HEK 293T SNCA KO complete cell lysate. Thrombin is a well-characterized target of thrombin-binding aptamers, which display high selectivity and specificity with little cross-reactivity to other sequences, and as such should not display reactivity towards the experimental aptamer of interest. BSA is rich in beta-sheet

specificity with little cross-reactivity to other sequences, and as such should not display reactivity towards the experimental aptamer of interest. BSA is rich in beta-sheet structures, similar to those found in amyloid proteins such as α-SYN, and serves to indicate whether or not the aptamer binds to a conserved structure rather than a specific protein, which would be undesirable. Finally, because the application of this split-system is to be in whole-cell environments, the aptamer will be evaluated for binding in a pool of physiologically relevant proteins, with the exception of α-SYN. Therefore, no signal should be detected upon incubation of the aptamer with the lysate sample[31]. Any significant cross-reactivity as indicated by the emission of a fluorescent signal, will result in repeating the aforementioned experiments using another aptamer sequence from the library of eight as reported by Ikebukro et. al.

Aim 1.2 Determine the Selectivity of the Split-System to Oligomeric vs. Monomeric α-SYN

A serial dilution of sonicated recombinant pre-fibrillar (PFF) and Monomeric α-SYN will be incubated with the split-constructs, and fluorescence intensity measured. The intensities of both monomeric and PFF will be compared to empirically determine the degree of selectivity of the sequence to PFF over monomeric. A native gel of each protein will be run as a control to ensure monomeric protein has not aggregated to a higher order state during storage in the event of significant cross-reactivity.

Aim 1.3 Establish a Calibration Curve for the Limit of Detection

A logarithmic series of α-SYN concentrates will be assayed with the split system. The lowest concentrations that produced a fluorescent response will be titrated similarly to determine the lowest amount of α-SYN necessary to produce a significant signal with

respect to signal: noise ratio. Monomeric α-SYN as well as total lysate will be analyzed at a fixed concentration as a control to optimize the signal: cross-reactivity noise as well.

Aim 2: The Ameliorative Effects of PDI in Live Cells Expressing Aggregated α-SYN

Aim 2.1 Cloning PDI into an Overexpression Vector System for Cytosolic Expression

Because PDI is typically maintained in the lumen of the endoplasmic reticulum, and α-SYN fibrillogenesis occurs in the cytoplasm, PDI expression needs to be redirected to the cytosol through transient transfection of a CMV promoter overexpression plasmid [36]. Each of the catalytic units of PDI will also be sub-cloned to evaluate which domain is enzymatically relevant for this experiment

Aim 2.2 Quantifying the anti-Fibrillogenic Properties of PDI Using a GLuc System

To semi-quantitatively ascertain if PDI has an effect on α-SYN aggregation, a Gaussian luciferase split-complementation system will be used. In this system, each half of a GLuc gene is linked to a gene coding for α-SYN[39]. Upon expression and challenge with Ferric Chloride ($FeCl_3$), α-SYN begins an aggregation cascade that brings the GLuc segments in close proximity thereby activating the luciferase catalytic domain; the degree of aggregation can be directly related to the luciferase signal. By triple-transfecting these two segments along with the inducible PDI system in HEK 293T SNCA KO cells, a time-dependent titer can be used to illustrate if PDI not only prevents fibrillogenesis but also reverses it, and at what stage of aggregation reversible is optimal. Negative controls include: SNCA KO transfected with the GLuc system and an empty vector with no PDI sequence, cells triple-transfected but not challenged with aggregation induced free-radical, and Cells transfected with the PDI construct but no GLuc system. In the event that the GLuc system does not suffice for evidence of aggregation, the cells can be lysed after treatment

and isolated protein fractions can undergo ultracentrifugation at >100,000xg to separate the aggregated and monomeric fractions[40]. The samples can be normalized, denatured, and run on a polyacrylamide gel so as to compare a change in amount of oligomeric species. ICC can also be performed to observe the absence of α-SYN punctations during the various PDI induction periods. In the event that a negligible effect of PDI is seen due to inefficient plasmid uptake or plasmid dilution, the inducible vector will also contain a puromycin resistance gene which will allow for the creation of a stable cell line with homogenous PDI expression.

Timeline

August 2016- October 2016

- Aim(s): 1.1, 1.2, and 2.1

October 2016- December 2016

- Aim(s): 1.3 and 2.2

Summary

In summation, this thesis serves to establish a foundation by which aptamer-mediated theranostics can be studied. Successful assaying, as well as amelioration of aggregated alpha-Synuclein can have a profound effect in Parkinson's disease translational research, potentiating the synthesis of a chimera to be used in conjunction with a blood-brain-barrier shuttling moiety to deliver a small, biologically compatible, non-immunogenic compound in vivo. This circumvents the need for inherently error-prone qualitative diagnoses, as well as

systemic therapeutics that result in a variety of secondary deficits, by focusing on the key molecular marker and mediator of Parkinson's disease: alpha-Synuclein.

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