CHAPTER 1

Introduction

1.1 Rationale

Alzheimer's is a neurodegenerative disease, which results in progressive cognitive impairment, including dementia, personality change, memory loss and death of the individual in the final. Alzheimer's disease is associated with the aggregation of amyloid-β (Aβ) peptide. The current research is focusing on the development of amyloid aggregation inhibitors that would impede the accumulation of amyloid oligomers and thereby slow the onset of the disease. Clinical trials for Alzheimer treatment have performed with the inhibitors of Aβ aggregation such as tramiprosate and scyllo-inositol (ELND005). However, these molecules failed in the clinical phases II and III because of their serious adverse effects such as low drug target, high toxicity in central nervous system (CNS) and statistically inconclusive results.

Recent researches are focusing on the using of natural medicinal plants for Alzheimer's treatment. These compounds were able to prevent neuronal damage and cell death. Nevertheless, their mechanism of action was not clearly identified. This thesis proposes to investigate the mechanism of phytochemicals on amyloid fibrillation by demonstrating how it influences the aggregation of amyloid fibril protein. Phytochemicals including plant-derived alkaloids, polyphenols and quercetin-iron complex were proposed as candidate molecules. The processes were first focused on the study of the interaction of phytochemicals with human recombinant insulin by using spectroscopic methods. Insulin was used as a model of amyloid fibrillation because its fibrils formation shares common mechanism with amyloid β aggregation. After characterizing their interaction, the effect of phytochemicals with amyloid-β protein, $A\beta_{40}$ and $A\beta_{42}$, were further determined as the mechanism of anti-aggregation.

Fluorescence spectroscopic method using Thioflavin T, a fluorescent dye used as stain for amyloid fibril formation, were used for monitoring the kinetic of amyloid fibrillation in the presence of phytochemicals. Finally, phytochemicals were performed *in vitro* using neuroblastoma cell lines, i.e. SH-SY5Y for assessing the neuroprotective effect on amyloid-β toxicity. The overall results of the thesis provided the information for future translational research and give a key molecule for the development of therapeutic drugs against Alzheimer's disease.

1.2 Literature Review

1.2.1 Protein folding and misfolding

Protein folding is an intrinsic feature of normal within the complex cellular environment. Its effects are minimized in living systems by the action of a protective mechanisms, including molecular chaperons and quality control system of cell [1, 2]. The native protein is reactive and stable. However, it is highly susceptible to change in the environment such as temperature, pH, ionic concentration, or surface energy [3-5]. These factors cause the protein to unfold and lose their function. If the denaturing conditions fall outside the limited stability range, a misfolded structure is produced [3]. The factor which enable denaturing conditions raises the internal energy of the protein and triggers the driving force to aggregate [3, 6].

In the last decades, many studies find out the main mechanisms of protein folding which is responsible for living control systems and cellular function [1, 7]. Conformational of protein, folding and unfolding, are associated with many cellular processes, including molecular trafficking, cell cycle regulations, and immune responses. The basis of different types of biological malfunctions is based on failure of protein misfolding, that resulting in various types of disease, most often characterized by the presence of the deposits of ordered protein aggregation in the affected tissues [7]. The function of misfolding proteins are inactive which lead to the loss-of-function features of the associated pathologies. This process can lead misfolded polypeptide chains to increase a toxic conformation, resulting in an aggregate form into ordered polymeric assemblies or to interact inappropriately with other cellular components, impairing cell viability and function, and eventually results in cell death [8, 9].

Therefore, the molecular basis of protein misfolding disease may ultimately be traced back to the presence of protein or peptide molecules with incorrect structures, different from those found in normally functioning organisms [10, 11]. The most important of protein misfolding diseases includes conditions whose molecular basis can be traced back to a toxic "gain-of-function", whereby specific peptides/proteins in their unstable, misfolded state self-organize into insoluble, cytotoxic, ordered and stable fibrillar polymeric assemblies with shared conformational features [12].

Indeed, protein misfolding and aggregation are ones of the most exciting new frontiers in protein science, cell biology and molecular medicine. Therefore, a better knowledge of the molecular basis of protein misfolding and aggregation may help elucidate the structural determinants and the physicochemical features of protein folding.

1) Protein aggregation and amyloids fibrillation

Native conformational aberration of some specific peptides or proteins can result in a wide range of human diseases. These pathological conditions are known to be related with protein misfolding [9]. More human peptides or proteins have been found to be misfolded and developed self-assemblies *in vivo*. These alterations can be characterized by conformational conversion of soluble proteins into insoluble amyloid-like fibrils [13]. The misfolded peptides or proteins associated with serious human amyloidogenic diseases including amyloid-β peptide (Aβ) in Alzheimer's disease, islet amyloid polypeptide in type 2 diabetes, α-synuclein in Parkinson's disease, and prion protein in the encephalopathies [14, 15]. The aggregation process is considered as a pathological phenomenon that depletes active proteins, and results in the formation of potentially toxic aggregated species of the cell [16]. Despite large differences in the size and native structure of amyloidogenic proteins, amyloid fibrils formed from different proteins display many commonalities. They are self-associating, highly repetitive polymers consisting of arrays of β-sheets that are held together via hydrogen bonding along the peptide backbone. The β-sheets are arranged parallel to the fibril axis in a cross-β fold. Amyloid fibrils are about 10 nm in diameter, but able to be extended up to several micrometers in length, twisted and unbranched morphologies [15, 17]

Amyloid fibrils arise when a specific protein or protein fragment converts from an otherwise soluble form into insoluble filamentous aggregates. Various amyloid-forming peptides and proteins exhibit the similar behaviors of aggregation. The kinetic of amyloid formation is generally initiated by “lag” or nucleation phase, followed by “growth” or a rapid exponential phase, and by a plateau phase in which no further polymerization occurs as shown in Figure 1.1[18, 19]. The lag phase is defined as the time for the formation of the soluble (prefibrillar) oligomers or nuclei. This phase begins with the destabilization and partial unfolding of the native protein, leading to the formation of an ensemble of intermediately folded species. Structural perturbation leading to protein unfolding can be achieved by exposing the native protein to one or more conditions, including elevated temperature, low pH, oxidative stress, molecular crowding, and protease-mediated degradation [18, 20]. A subset of proteins within this ensemble will undergo non-ordered aggregation to form nuclei. After nucleation and immediate preceding the polymerization phase, the nuclei will transform into an ensemble of various assemblies called protofibrils that exhibit increased levels of complexity. During the polymerization phase, these protofibrils rapidly grow to form well-ordered protofilaments which laterally associate to rise to mature amyloid fibrils [18, 21].

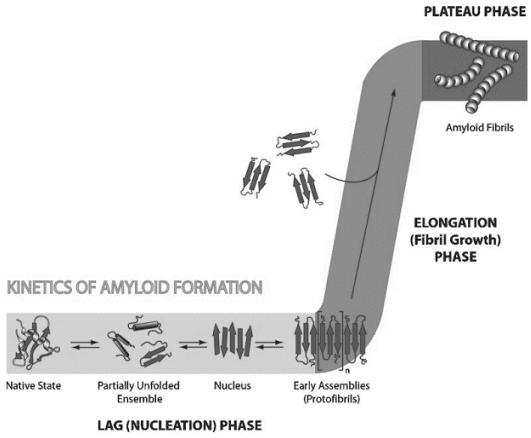

Figure 1.1 A schematic representation of the amyloid forming pathway [18]

1.2.2 Alzheimer's disease: a neurodegenerative disease-causing protein misfolding and aggregation

Alzheimer's disease is currently an incurable neurodegenerative condition which is highly prevalent in elderhood. In 1906, Alois Alzheimer was the first psychiatrist and neuropathologist who analyzed brain tissue from a patient who died from an unknown mental illness [22]. According to the Alzheimer's Association, Alzheimer's disease is the fifth leading cause of death in patients at the age over 65 years old, 13% of people encountered from this disease in developed countries, [23].

1) Mechanism of Alzheimer's disease

The mechanism of Alzheimer's disease is associated with the accumulation of insoluble forms of amyloid-β in extracellular spaces, as well as in the walls of blood vessels, and the aggregation of the microtubule protein tau in neurofibrillary tangles in neurons [24]. However, it suggests that Aβ accumulation play an important role in Alzheimer's disease pathogenesis [25]. Aβ is derived by the proteolytic cleavage of amyloid precursor protein (APP) by a family of enzyme (γ-secretases and β-secretases), which includes Presenilin 1 (PS1; encoded by PSEN1) and Presenilin 2 (PS2 encoded by PSEN2) [24]. Cleavage of amyloid precursor protein released the amyloid β peptide into the extracellular space as diffusible oligomers ($A\beta_o$) [26]. $A\beta_o$ can be eliminated by mechanisms that involve APOE or can be taken up by astrocytes via low-density-lipoprotein-receptor-related protein 1 (LRP1) [27]. $A\beta_o$ can also aggregate into fibrillary constructions, which turn assembly into plaques. Aβ plaques can be eradicated from brain via degradation by endocytic or phagocytic clearance, or by endoproteases from astrocytes [28, 29]. However, some conformational oligomers that dissociate from Aβ fibrils and plaques may not be cleared and are toxic to adjacent synapses and induce tau aggregation. Tau damage occurs in neurons and is mediated by the development of tau-positive neurofibrillary tangles. Fibrillar tau can be released and taken up by healthy neurons, triggering tau damage in the uptaking cell [30]. In addition, Aβ oligomers might drive α-synuclein aggregation in the plaques. Besides, mitochondrial damage or dysfunction might also be involved in the neurodegenerative process [31].

2) Role of amyloid-β in Alzheimer's disease

Aggregation of amyloid β plays a key role in the pathogenesis of Alzheimer's disease. Aβ is a proteolytic product of amyloid precursor protein by β- and γ-secretases [24]. The imprecise cleavage of γ-secretase at C-terminus of Aβ sequence results in two major Aβ isoforms: $A\beta_{42}$ (42 residues long) and $A\beta_{40}$ (40 residues long). The sequences of $A\beta_{40}$ and $A\beta_{42}$ are quite similar, the only difference is an extra isoleucine and alanine at the C-terminus of $A\beta_{42}$ [32, 33]. $A\beta_{42}$ is the major component of amyloid plaques in AD brains, while $A\beta_{40}$ is detected only in a subset of plaques [34, 35]. $A\beta_{42}$ is more amyloidogenic and form fibrils significantly faster than $A\beta_{40}$ [19, 36]. $A\beta_{42}$ has higher cellular toxicity, and more directly related to the AD pathology. The prominent neurotoxin in AD was a $A\beta_{42}$ dodecamer [37]. Therefore, $A\beta_{40}$ and $A\beta_{42}$ may have different connections and influences on the AD pathology. Recent clinical experiments suggest that $A\beta_{42}/A\beta_{40}$ ratio was more relevant to the AD pathogenesis than the amount of $A\beta_{42}$ or $A\beta_{40}$ [38, 39]. The mutation of APP and secretase genes is thought to induce the elevation of $A\beta_{42}/A\beta_{40}$ ratio, and finally results in the early onset AD [40, 41]. The $A\beta_{42}/A\beta_{40}$ ratio could be a biological indicator for the AD diagnosis [42]. In consequence, the development of selective probes or inhibitors of $A\beta_{42}$ or $A\beta_{40}$ for eliminating a certain type of Aβ isoform should be investigated.

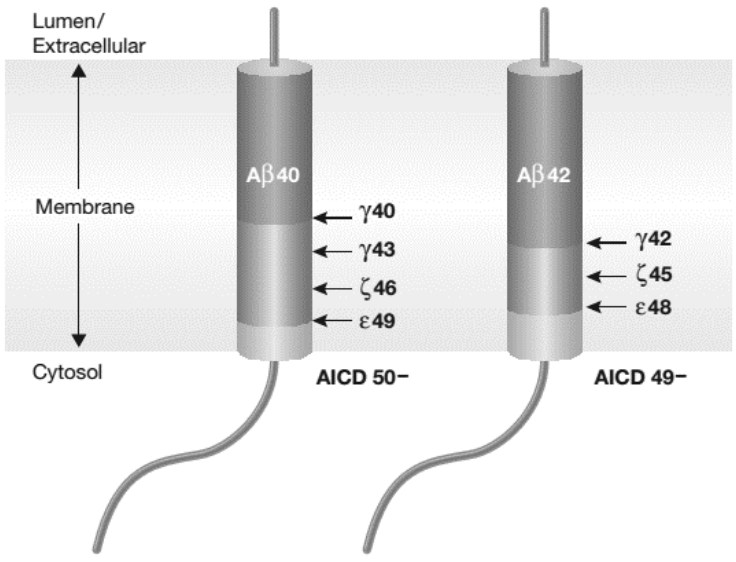

Figure 1.2 Characteristics of $A\beta_{40}$ and $A\beta_{42}$ protein [43]

3) Toxicity of amyloid Beta protein

Despite various isoforms of Aβ, with differing propensities for aggregation, three major groups of Aβ assemblies exist comprised of monomers, soluble oligomers, and insoluble fibrils which have been termed as “Aβ pools”. Each pool encompasses multiple structures of Aβ aggregation based on various organizations [43]. Soluble Aβ oligomers have been reported in AD to be organized into different structures ranging from dimers, trimers, tetramers, pentamer, decamers, Aβ-derived diffusible ligands (ADDL), dodecamers, and Aβ*5[37, 44-47].

There are factors that distinguish toxic soluble oligomers from the monomers or higher aggregates, such as fibrils, and identification in AD brains. It is suggested that an inverse correlation should be between the size of Aβ assemblies and the potency of their exerted toxicity. As the size of the oligomeric assembly increases, its deleterious effects decrease as shown in Figure 1.3 [48]. Aβ dimers have been shown to assembly form a more stable structure of higher molecular weight, termed protofibrils which are neurotoxic. Thus, dimeric units of Aβ have been considered to be an important entity providing the building blocks for the toxic aggregates [49, 50].

Membrane disruption and ion dysregulation are known to be components of Aβ toxicity. Aβ oligomers have also been shown to directly interact with membranes forming pores and disrupting the proper permeability of membranes [51]. Aβ oligomers may sensitize the mitochondrial permeability transition pore to Ca^{2+} resulting in mitochondrial dysfunction and cell death [52].

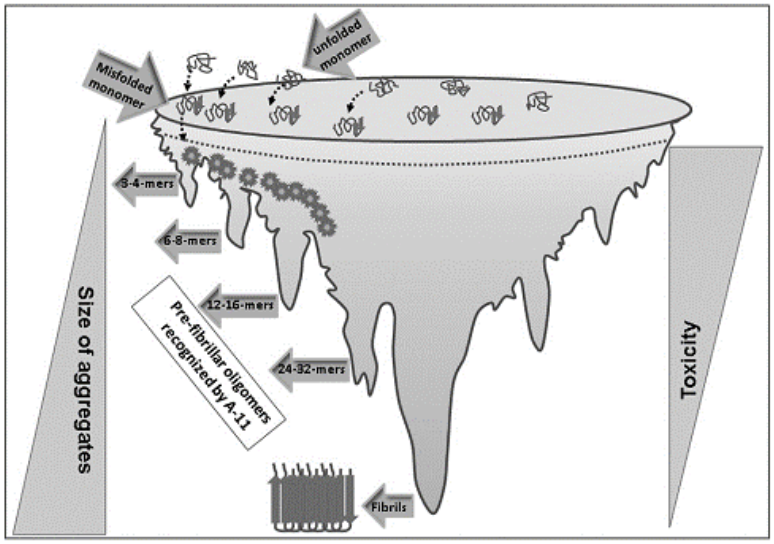

Figure 1.3 The relationship between the size of Aβ and their toxic effects [49]

1.2.3 Therapeutic approaches against protein aggregation in Alzheimer's disease

The incidence of Alzheimer's disease has been growing continuously. Finding an effective treatment became more important. Amyloid β has been being the focus of research for several decades. An ever-growing incidence of AD has led researchers and clinicians to discover the cure. Therefore, the finding of the novel therapeutic approaches will pave the way for AD treatment.

1) Inhibition of β-amyloid peptide aggregation by clinical trial drugs

Aβ aggregation give rise to amyloid plaques and then, developed into senile plaque formation. The only inhibitor of Aβ aggregation that reached phase III trials is 3-amino-1-propaneosulfonic acid (3-APS, Alzhemed, Tramiprosate) [53]. This inhibitor was designed to antagonize the interaction of soluble Aβ with endogenous glycosaminoglycans which promote aggregation of Aβ into amyloid fibril formation and deposition [53]. However, the disappointing results of the phase III clinical trial in 2007 have led to the suspension of this compound in Europe [54]. Colostrinin, a complex of proline-rich polypeptides present in ovine, bovine, and human colostrum, inhibits aggregation of Aβ and its neurotoxicity in cell assays, and improves cognitive performance in mice models [55]. Although a phase II trial showed slight improvements in Mini Mental State Evaluation in patients with mild AD in a treatment period of 15

months, this beneficial effect was not maintained after another 15 months of continuous treatment [55]. Scyllo-inositol (ELND005), an oral amyloid anti-aggregation agent, is capable of decreasing Aβ toxicity in the mouse hippocampus. ELND005 was conducted in participants with mild-to moderate AD in 18-month long phase II clinical trial. This dose-finding, safety, and efficacy trial did not meet its primary clinical efficiency outcomes [56]. Clinical trials for AD treatment were also performed with metal chelating 8-hydroxiquinolines (8-HQ) compounds, clioquinol and PBT2 [57]. These molecules block the interaction between the base metals and brain Aβ peptide. It is shown that increased levels of oxidative stress in the brain of AD patients might be partially depend on copper ions binding to Aβ, leading to metal-mediated generation of reactive oxygen species (ROS) [58-60]. It was also hypothesized that 8-HQs may prevent Aβ aggregation while simultaneously restoring homeostasis in cellular levels of copper and zinc ions [59, 60]. Unfortunately, these molecules failed in the phases II and III of clinical development due to lack of efficacy.

2) Phytodrugs: a potential alternative against protein aggregation in Alzheimer's disease

Recently, more efforts are being focused on a great number of natural medicinal plants for therapeutic properties test. Showing that the raw extracts or isolated pure compounds from natural medicinal plants have more effective properties than the whole plant, as an alternative for the treatment of ND. These properties are depending mainly to the presence of polyphenols and alkaloids. These compounds can prevent neuronal damage and cellular death [61]. Thus, these natural compounds can be used in the treatment of AD and able to be models for developing new specific drugs against AD pathologies.

Alkaloids are a class of naturally occurring organic nitrogen-containing compound extracts. Alkaloids represent a wide and ancient family of compounds with analgesic, anti-asthmatic, antiarrhythmic, anticancer, antihypertensive, antipyretics, antibacterial and antihyperglycemic activities [62]. Since the 1960s, the role of alkaloids in the field of dementia has been extensively investigated. The Food and Drug Administration's approval for the two alkaloid-based drugs, galantamine and

rivastigmine, for AD treatment in the early 2000s has led to a renewed interest in alkaloids for dementia therapy [63]. Matharu and Melo found that galantamine inhibits Aβ aggregation and cytotoxicity [64, 65]. In addition to galantamine, it was found that berberine inhibits the formation of pathogenic Aβ, and Aβ aggregation in neuronal cells stably expressing human Swedish mutant APP [66, 67]. Caffeine reduced levels of Aβ and Aβ-induced neurotoxicity both *in vitro* and *in vivo* as well as improves cognitive performance in Aβ induced AD mouse models. Caffeine also reduces levels of Aβ in neuroblastoma-2a cells, stably expressing human Swedish mutant, and protects cerebellar granule neurons and basal forebrain neurons from neurotoxicity caused by Aβ [68]. As known, alkaloids have displayed a trend of efficacy in the management of AD. Contrastingly, they have also shown side effects, such as toxicity and addiction properties.

Polyphenols are a large group of natural and synthetic small molecules which are composed of one or more aromatic phenolic rings. Several polyphenols from natural products such as red wine, grapes, green tea, and turmeric have been reported to inhibit protein aggregation and toxicity both *in vitro* and *in vivo* [69]. Curcumin has been widely investigated for its anti-aggregation properties in many proteins including amyloid-β, synuclein, transthyretin, lysozyme and insulin etc. [70, 71]. It is possible that it may cross the blood brain barrier due to its lipophilic nature and binds to amyloids that lead to attenuation of their cytotoxicity. Many studies advocate curcumin as potential therapeutics in AD. It inhibits amyloid formation, and destabilizes fibrillar or oligomeric forms [72]. Curcumin also binds to monomeric alpha synuclein suppressing aggregation, and increment in reconfiguration rate [73]. Epigallocatechin-gallate (EGCG) a member of flavonols family and is mostly found in green tea. It decreases the amyloid beta aggregation and plaque deposition in the brain of Alzheimer's transgenic mice [74]. Resveratrol dose dependently inhibits fibril formation of amyloid beta. It also reduces the amyloid induced cytotoxicity in neuronal cell lines [75, 76]. Tannic acid inhibits amyloid beta aggregation and it is also the most potent inhibitor of prion protein [77]. Red wine polyphenols (catechin, epicatechin, myricetin, kaempferol and quercetin) are also reported to inhibit the aggregation of many proteins including amyloid β, alpha synuclein and lysozyme etc.[78, 79].

1.2.4 An approach for investigating amyloid protein fibrillation

1) Insulin: a model system for the study of amyloid fibrillation

One of the proteins where amyloidogenesis is of potential concern is insulin. Insulin plays a major role in blood glucose homeostasis and commonly be used to treat diabetes. Insulin, in its native conformation, is a small polypeptide hormone composed of 51 amino acids with a large α-helical structure [80]. It is produced by pancreatic β cells and stored predominantly as zinc-coordinated hexamers in the secretory granules within pancreatic islets. When released into bloodstream, insulin in a monomeric form binds to its receptor for regulating glucose metabolism in biological systems [81]. The driving forces lead to dimerization are predominantly non-polar, reinforced and given direction by the antiparallel β sheet of hydrogen bonds. Under appropriate conditions (higher insulin concentration, basic pH, and presence of zinc ions), three dimers associate to form a stable torus-shaped hexamer, in which both polar and non-polar residues are buried between the dimers.

Insulin is an important model system for understanding protein aggregation and amyloid protein fibrillation. Under specific conditions (low pH, high temperature, and higher ionic strength), insulin can be triggered to rapidly form amyloid fibrils *in vitro* [83]. Insulin fibrils formation share a common structure with other amyloidogenic proteins, insulin has been widely used as a suitable model system to understand the pathological mechanism of protein misfolding diseases [84, 85]. For this reason, insulin was used extensively to understand the physical chemistry of aggregating systems.

Similar to other amyloidogenic proteins, insulin fibrillation is proposed to occur under various conditions via the aggregation of partially folded intermediates through a nucleation mechanism [86, 87]. The kinetic process of fibril formation in experiments is commonly characterized as an apparent lag period followed by an exponential growth regime, and a final plateau regime, as shown in Figure1.4.4. The lag period is mainly due to a reversible nucleation process, in which the amount and size of insulin aggregates form are not enough to be detectable. Once the concentration of nucleation reaches its critical state, the exponential growth occurs,

which subsequently elongates the nuclei into long unbranched fibrils, leading to an irreversible formation of large fibrils. Finally, when the concentration of insulin falls below the threshold, a stable plateau regime is achieved, aborting further fibril extension [80].

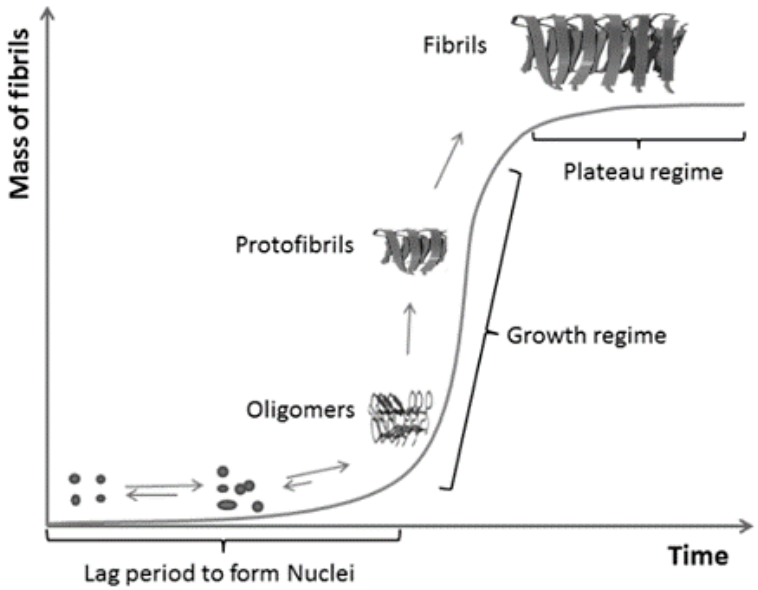

Figure1.4 A typical kinetic graph of the insulin aggregation model [81]

2) Tyrosine autofluorescence: an intrinsic protein fluorescence

Intrinsic protein fluorescence is a sensitive technique that has been exploited in studying the structural, physicochemical, and functional properties of proteins. Proteins derive their intrinsic fluorescence from the chromophores phenylalanine, tyrosine, and tryptophan. Insulin contains four Tyr and three Phe residues. In the absence of Trp, Tyr dominates the absorption spectrum of proteins to the exclusion of Phe and nonaromatic absorption attributable to cystine, histidine, or the peptide bond. It has been found that protein denaturation results in marked uniformity in its fluorescence characteristics. It is interesting to note that structural changes in proteins can be detected via intrinsic protein fluorescence which can be applied in resolving the complex structural transitions involved in protein aggregation. The main advantage of using intrinsic protein fluorescence is that it provides information on the dynamics associated with protein aggregation at the molecular level. To probe the kinetics of insulin fibrillation via intrinsic fluorescence, it is important to appreciate the

underlying cause of the observed Tyr fluorescence quenching and how this feature is related to the fibrillating protein [88].

3) Thioflavin T as probe for monitoring amyloid protein formation

Thioflavin T (ThT) is the most widely used as amyloid dye among the hundreds of amyloid reports published. ThT is a commonly used probe to monitor *in vitro* amyloid fibril formation according to its broad staining capacity, extraordinary sensitivity, and ease of use [89].

Upon ThT bind to amyloid fibrils, which gives a fluorescence emission at approximately 482 nm when excited at 450 nm. The mechanism of fluorescence enhancement has been attributed to the rotational immobilization of the central C–C bond, connecting the benzothiazole and aniline rings [90, 91]. The dramatic increase of ThT fluorescence results from the selective immobilization of a subset of ThT conformers [92]. Benzylamine and benzathiole rings of ThT rotate freely near their shared carbon-carbon bond in a solution with a low energy state. This rotation rapidly quenches excited states generated by photon excitation, causing low fluorescence emission for free ThT. In the excited state, rotational immobilization of ThT was preserved, resulting in a high quantum yield of fluorescence. Amyloid fibrils are likely to present a ThT-binding site that sterically “locks” the bound dye, thus leading to an enhancement of ThT fluorescence [89]. In addition, ThT has also been shown to bind to a hydrophobic pocket of human serum albumin with comparable affinity to many drug-like molecules [93]. ThT’s capacity to interact with hydrophobic pockets in non-fibrillar proteins may also rationalize its ability to bind in cavities between protomers of insulin fibrils [94].

1.3 Objectives

The objectives of this thesis were

1.3.1 To characterize the interaction between phytochemicals and human amyloid protein by using spectroscopic techniques.

1.3.2 To investigate the effect of phytochemicals on amyloid fibrillation.

1.3.3 To determine the neuroprotective effect of phytochemicals on amyloid fibril toxicity *in vitro*.