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Chapter 1

Introduction

One of the most remarkable phenomena in nature is the ability of proteins to fold from linear polypeptide chains into structures which impart their functions as molecular machines of life. Proteins play a key role in all aspects of life, from cell cycle regulation to signal transduction. Much of the critical regulation of biological activity within a cell is mediated by binding interactions between a protein receptor and an inhibitory ligand. For example, about 40% of modern medicine targets G protein-coupled receptors, a family of proteins found in eukaryotes that sense molecules outside of the cell and induce cellular responses by activating intracellular signal transduction pathways. Hence, it is not surprising that the improper functioning of proteins due to mutations, denaturation, or misfolding (failure to adopt their native functional state) cause many diseases.

A seminal experiment performed by Anfinsen and colleagues in 1961 demonstrated that the structure of a folded protein is encoded in its amino acid sequence and solvent environment. Since then, much progress has been made in understanding protein structure, function, and mechanism of folding. Structure determination techniques have gained much attention in the fields of biochemistry and biophysics, and have provided valuable insights into macromolecular structure. However, protein structures determined from NMR, X-ray crystallography, and homology modeling only provide a static picture,

whereas molecular recognition and drug binding are very dynamic processes. When a substrate approaches its receptor in solution, it encounters not a single, frozen structure, but rather a macromolecule in constant motion. Understanding both protein structure and dynamics enable us to elucidate the molecular basis of disease pathways, which will ultimately contribute to the discovery of novel therapeutics.

Because ligand binding and the important macromolecular motions associated with it are microscopic events that take place on the scale of millionths of a second, a complete understanding of the atomistic energetics and mechanics of binding is unattainable using current experimental techniques. In recent years, molecular dynamics (MD) simulation of biomolecules, a physics-based computer simulation technique, became a tool of choice to investigate protein dynamics and function, and ligand-binding. Currently, MD simulation is the most accurate computational method for probing small-molecule binding, and is useful for filling in the details where experimental methods cannot.[1] In the past few years, MD simulation studies have been routinely reaching microseconds in sampling time. With the ever increasing availability of computing power and data storage, simulations are a promising technique for aiding in the structure-based drug discovery process.

In recent years, a class of proteins without a uniquely folded state known as intrinsically disordered proteins (IDPs) have gained attention because of their involvement in a multitude of physiological pathways and diseases. For example, proteins associated with cell signalling and cancer in humans are predicted to be enriched in protein disorder.[2] A detailed review of disordered proteins and their roles in biology is beyond the scope of this thesis and is provided elsewhere.[3,4] A number of IDPs are able to self-aggregate to form amyloid, and are associated with incurable diseases such as prion disorders, neurodegenerative diseases, Type II diabetes, and systemic amyloidosis.

1.1 The amyloid state of proteins

Amyloids were discovered 150 years ago when tissue deposits of extracellular filaments were observed.[5,6] These microscopically visible deposits were found on various organs in many seemingly unrelated diseases. Although numerous diseases involve amyloid formation of distinct aggregation-prone proteins or peptides, the ability to form amyloid is not only restricted to these disease-associated proteins. Amyloid fibrils may be formed from proteins with well-defined tertiary structures (eg. myoglobin and lysozyme), suggesting that the ability to form amyloid fibrils may be a generic property of polypeptides.[7] However, the propensity for a given protein or peptide to form amyloid is highly dependent on the solution condition and peptide sequence. For example, for a globular protein to form amyloid, the protein must first be partly unfolded before conversion into amyloid fibrils is possible.[7]

The formation pathway of amyloid fibrils in vivo is not understood. Much of what we know about amyloid formation currently comes from biochemical and biophysical analysis of synthetic amyloid-forming peptides in vitro, which is thought to be analogous to the in vivo pathway. Prior to the appearance of amyloid fibrils, a variety of intermediate species may be formed. Monomers self-assemble into oligomers of different morphologies and sizes, which exist in equilibrium with amyloid fibrils, a visible endpoint of aggregation.

Kinetically, the mechanism of amyloid formation is akin to those of nucleation-polymerization processes such as crystallization and micelle formation.[8] During nucleation, a lag phase occurs where the energetic barriers of aggregation must be overcome by the monomers to form the initial aggregation nucleus.[8] Following this lag phase, free monomers may bind to the nucleated aggregates, which elongate into mature fibrils.[8] Seeding, a process where preformed aggregates are introduced into the solution, eliminates the lag phase.[9,10] In the sections below the current biophysical and structural data on the structure of amyloid fibrils and non-fibrillar oligomers is reviewed, and implications for amyloid disease are discussed.

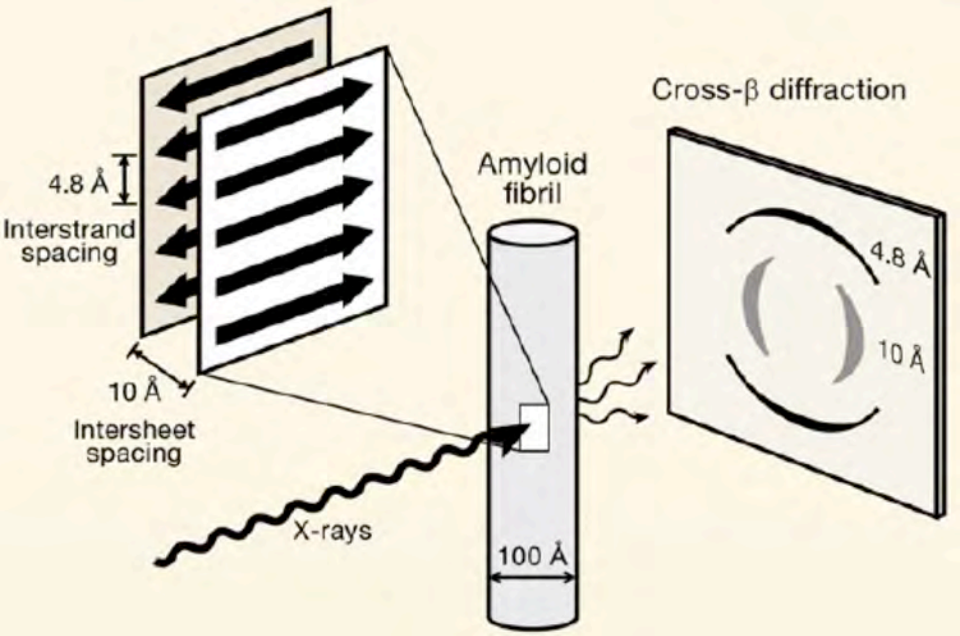

Figure 1.1: A schematic of the cross-β structure and the diffraction pattern of fibrils. (Adapted from Eisenberg et. al., 2012)

1.1.1 Fibrils

Fibrillar amyloid deposits have several physical properties in common. Fibrils are protease resistant, and insoluble in the presence of the detergent sodium dodecyl sulfate.[11] Importantly, they exhibit specific optical behavior when bound to certain dye molecules. After staining with Congo red, fibrils exhibit bright green birefringence under polarized light. However, the use of Congo red to detect the presence of amyloid formation is often a laborious process, and only provides a qualitative measurement of the amount of amyloid present. Thioflavin-T (ThT), a benzathiole fluorescent dye, is more commonly used to detect the presence of amyloid fibrils in post-mortem brain tissue samples, and monitor fibril formation in vitro. Upon binding to fibrils, ThT exhibits a dramatic shift in the excitation spectrum maximum and an emission enhancement, making it a sensitive and efficient reporter for the presence of amyloid fibrils.

Amyloid fibrils formed from different polypeptides all share a similar morphology known as the cross-β structure. To date, independent measurements of fibrillar structure

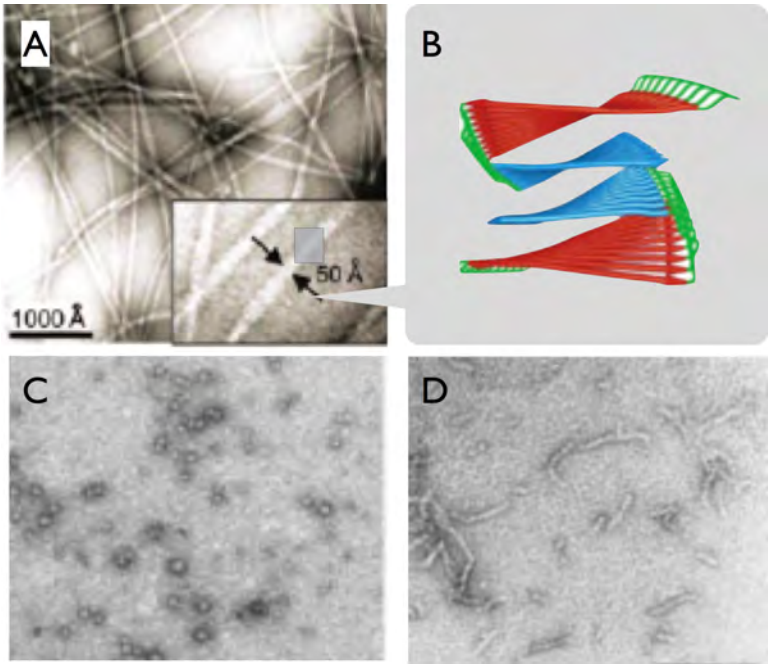

Figure 1.2: (A) TEM image of negatively-stained mature amyloid fibrils. (B) SSNMR model proposed by Tycko et al. (C) and (D) EM images of oligomers of Aβ40 adapted from Bitan G. et al. 2003 and Walsh D. 1999. **ADD the fibril axes.**

from different instruments have all confirmed the cross-β as the core structure of amyloid fibrils. X-ray fiber diffraction studies showed that the diffraction pattern of fibrils is characterized by major orthogonal reflections along the meridional and equatorial directions, which correspond to a 4.8 Å interpeptide separation, and a 10 Å intersheet separation, respectively (Figure 1.1).[6,12,13] The inter-peptide, and inter-sheet separations are respectively parallel and perpendicular to the long-axis of the fibril. This diffraction pattern is now considered as indicative of the presence of cross-β structure, and hence, of amyloid fibrils. When stained, the macromolecular morphology of fibrils can be determined using the transmission electron microscope (TEM): fibrillar structures are long, unbranched, and ribbon-like structures with diameters between 50 - 100 nm (Figure 1.2).[7]

Advances in solid-state NMR (SSNMR) and X-ray crystallography in the last decade have elucidated the molecular details of amyloid fibrils. One of the first SSNMR model of an amyloid fibril was that of Aβ40, a peptide implicated in Alzheimer's Disease.[14] Its

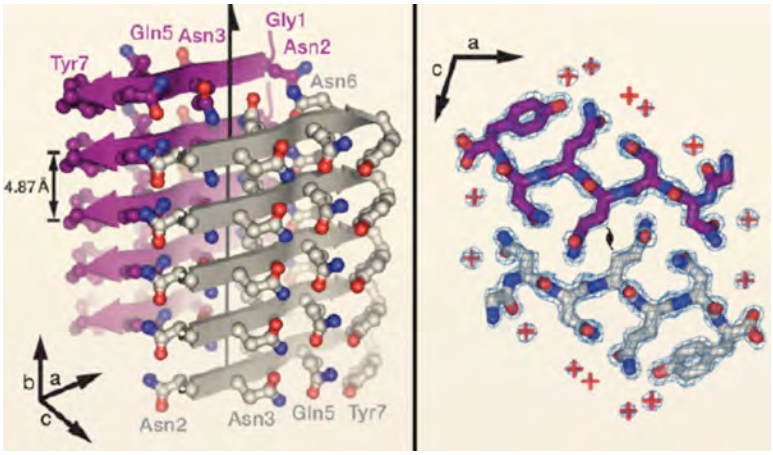

Figure 1.3: A schematic of the X-ray crystal structure of fibrils formed from short amyloidogenic peptide fragments. Adapted from Eisenberg, 2012

core fibril unit consisted of a parallel in-register β-sheet, where each strand is a β-hairpin with peptide-peptide backbone hydrogen bonds running in parallel to the long-axis of the fibril (Figure 1.2). Moreover, smaller fragments of Aβ have been shown to form fibrils that are morphologically similar to those of the full length peptide. For example, SSNMR studies of the fibrils of the peptide Aβ(16-22) or KLVFFAE, the central hydrophobic core of Aβ, indicated that its fibrils are composed of stacked antiparallel β-sheets.[15] Fibrils of certain amyloid-forming peptide fragments formed crystals that were amenable to single crystal X-ray diffraction analysis.[11] In agreement with SSNMR, the crystal structures of these fibrils revealed a structure composed multiple layers of β-sheet with a dehydrated ("dry") stacking interface (Figure 1.3).[11,16]

Depending on the experimental conditions under which they are formed, fibrils may exhibit polymorphism: the length of the β-strand involved, side chain orientations and inter-protofilament packing may vary.[17] Fibril polymorphism may have important implications in amyloid diseases because, depending on which residues are exposed at the surface, different morphologies exhibit differing toxicities. For example, in vitro, quiescently formed fibrils of Aβ(1-40) have been shown to be more toxic than agitated fibrils.[18]

1.1.2 Non-fibrillar oligomers

Because of their structural disorder and transient nature, obtaining high resolution structural details of amyloid oligomers using traditional structural determination techniques have been impeded. EM and AFM experiments have shown that transient, unstable particles may appear prior to the formation of fibrils ("on-pathway" oligomers).[19–21] Those that do not progress to form fibrils are considered as off-pathway.[22] However, off-pathway oligomers formed in the presence of detergents, lipids, and certain small molecules are typically not considered to be biologically-relevant.[23,24]

Unlike fibrils, amyloid oligomers do not possess a generic structural element and instead, adopt a wide spectrum of sizes and morphologies. Size exclusion chromatography (SEC) studies of Aβ40 oligomers (isolated in vitro and from brains of deceased individuals with Alzheimer's Disease) showed dimers to large oligomers with hundreds of peptides.[5,25] Oligomeric assemblies that are annular, spherical, or curvilinear in shape have been reported in literature.[5,26,27]

Although there are large variations in morphologies, oligomers formed from different polypeptide sequences can display similar activities in cell metabolic assays.[28] Importantly, many oligomers share the ability to interact with an oligomer-specific antibody.[22] Several studies have indicated that oligomers may possess high β-sheet content.[20,29,30] Moreover, some non-fibrillar oligomers may contain common structural elements: high-resolution structural studies of non-fibrillar oligomers of Aβ42 and prion-like peptides suggest that they may contain cross-β like fragments.[29,31,32]

1.2 Amyloid involvement in diseases

Because numerous diseases shared the amyloid plaque pathology, fibrils were initially hypothesized to be the toxic species in these diseases. However, recent research has implicated non-fibrillar oligomers as the more likely toxic agent in the cause of sev-

eral neurodegenerative diseases, and type II diabetes.[5,33,34] Currently, the mechanism of toxicity of amyloid oligomers has not been determined, and is an area under intensive research. Oligomers formed from a variety of peptides, including those not implicated in amyloid disorders (e.g. lysozyme, β2-microglobulin, transthyretin) all exhibited toxicity, suggesting that the toxicity of amyloid oligomers may be independent of the peptide sequence.[22,35] Experimental evidence widely supports that this generic mechanism of toxicity is due to the ability of oligomers to interact with the cellular membrane.[25,36] It is hypothesized that oligomeric aggregates may ultimately induce cell death by interacting with and disrupting the integrity of the cellular membrane.[35] Moreover, the aggregation of amyloidogenic peptides was found to be enhanced on membrane surfaces,[37–39] and that membrane-catalyzed fibril formation may induce cellular toxicity.[40]

1.3 Alzheimer's Disease

Alzheimer's Disease (AD) is a devastating neurodegenerative disease that is the most common cause of dementia in persons of age 65 or older. Upon examination, the post-mortem brains of AD patients show significant neuronal dystrophy. Pathologically, AD is characterized by the presence of extracellular deposits of senile plaques and neurofibrillary tangles (NFTs), both of which appear as lesions on stained neuronal tissue under light microscopy (Figure 1.4).

In 1985, the amyloid-β protein or Aβ was identified as the largest component of these plaques.[41] Monomeric Aβ is an approximately 4 kDa peptide produced by the intramembrane proteolytic cleavage of a larger protein, amyloid-β precursor protein (APP).[42] APP is sequentially processed by the aspartyl proteases β-secretase and γ-secretase, where depending on the position of the cleavage by γ-secretase, a pool of Aβ peptides of lengths varying from 38 to 43 residues are produced (Figure 1.5).[43] The peptides spanning residues 1-40 (Aβ40) or 1-42 (Aβ42) are predominantly found in AD-associated

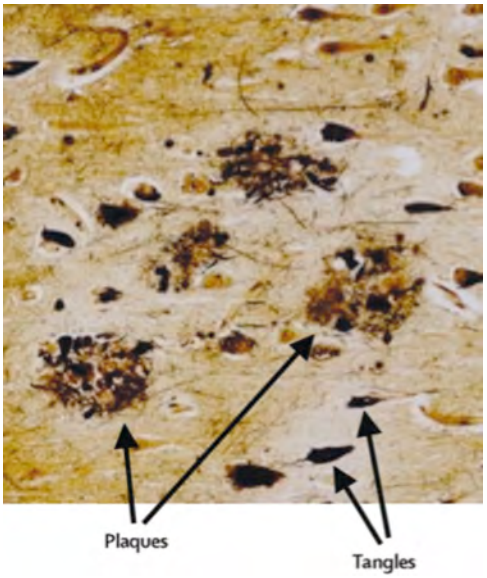

Figure 1.4: Lesions formed from amyloid plaques and NFT tangles in the cerebral cortex tissue of an AD brain. Adapted from Blennow, 2006

plaques.[44,45] Although plaques contain different isoforms of the Aβ peptide, Aβ42 is likely to be the more deleterious form of Aβ. In vitro, Aβ42 displays significantly higher propensity for aggregation than Aβ40, despite differing by only two amino acids.[46–48] Furthermore, genetic mutations within presenilin 1 and 2 (genes encoding enzymes that cleave APP to produce Aβ peptides) cause an aggressive early-onset form of AD which also lead to an increase in the ratio of Aβ42 to Aβ40 peptides produced.[49–51]

Although it has been more than one hundred years since Alois Alzheimer first associated the presence of neuronal plaques with the clinical symptoms of Alzheimer's disease (AD), the exact relationship between the two is still under much contention. The ubiquitous presence of amyloid plaque deposits found in the brains of deceased dementia patients led to the formulation of the long-standing amyloid cascade hypothesis: the amyloidogenesis of Aβ plays a key role in the initiation of AD, which ultimately leads to the clinical symptoms of dementia.[42] Genetic evidence provided strong support for the amyloid hypothesis: in those with trisomy 21 (occurring in Down's syndrome), the chromosome responsible for encoding APP, the overproduction of Aβ leads to early-onset

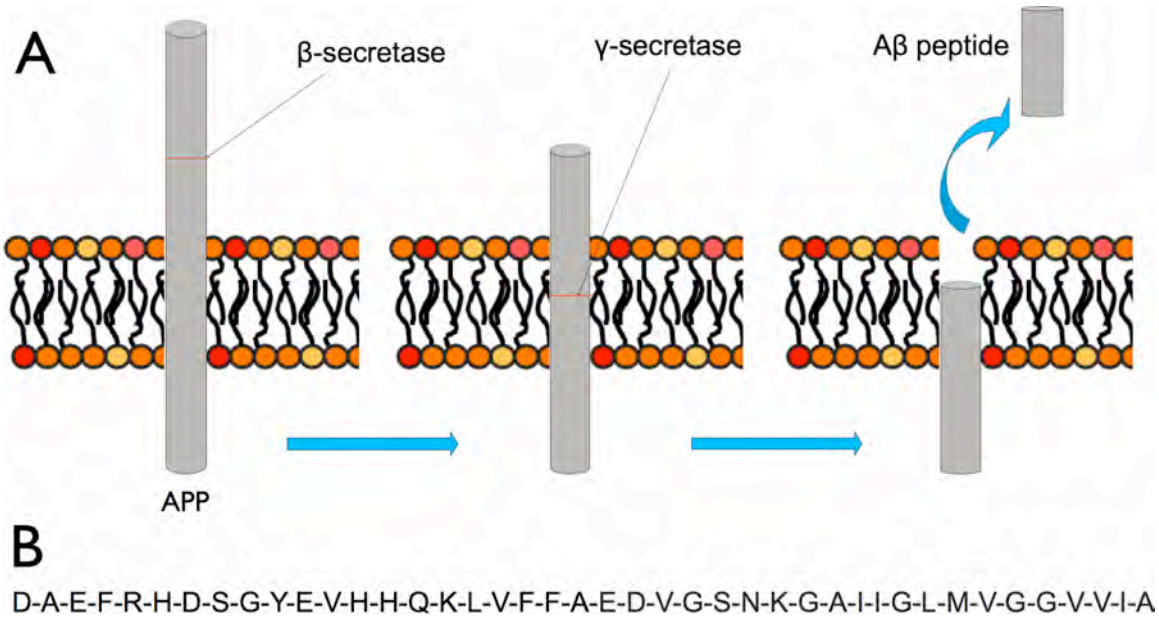

Figure 1.5: A schematic of the production of Aβ via the proteolytic processing of amyloid precursor protein is depicted in (A). The peptide sequence of Aβ42 is shown in (B).

of dementia with AD-like plaque load. Furthermore, in persons with early-onset familial AD, genetic mutations on the APP lead to the production of Aβ peptides with increased aggregation propensities.[52]

In addition to the presence of amyloid plaques, another hallmark of AD is the intracellular deposition of neurofibrillary tangles (NFTs) composed of aggregated hyperphosphorylated forms of the microtubule-associated protein tau.[53] The role of the tau protein and its interactions with amyloid in the pathogenesis of AD are still being established.[54] Studies with mouse models currently suggest that the role of NFTs in AD may be downstream to that of Aβ: Aβ plaque pathology was not developed in a tau transgenic mouse model, whereas Aβ formation in APP transgenic mice was found to induce hyperphosphorylation of tau.[55]

A puzzling aspect of AD is that the plaque load in the brain of dementia patients is often not correlated with their disease progression and severity. Instead, multiple lines of evidence indicate that synaptic loss and the severity of cognitive impairment are correlated with the concentration of soluble Aβ oligomers in the brain.[56–58] For example,

oligomers extracted from AD brain can impair synapse structure and function.[59] Moreover, when injected into the brains of animal models of AD, Aβ oligomers decreased the number of synapses and impaired learning performance.[52,60–62] Furthermore, cellullar models of toxicity displayed characteristic symptoms of neurotoxicity that lead to eventual apoptosis upon the addition of Aβ oligomers prepared either in vitro or extracted from cell cultures.[25,59,63–65] Taken together, current experimental evidence indicates that preventing the formation of oligomeric forms of Aβ may be a promising method of treatment for AD.

1.4 Amyloid Inhibition by small molecules: A promising method of treatment for AD

With the longevity of our population, AD is approaching epidemic proportions with no cure or preventative therapy available.[66] In 2010, it was estimated that 36 million people in the world were suffering from AD, and is projected to grow to 115 million people by the year 2050.[67] Furthermore, there are no drugs which may target the underlying disease: approved treatments today such as donepezil (a cholinesterase inhibitor), and memantine (a N-methyl-D-aspartate antagonist) only mitigate cognitive symptoms.[68]

Hence, it is imperative that efforts be made toward the development of therapeutics for AD. The vast number of structural and biochemical studies on amyloid structure have been crucial for the development of potential therapeutics for treating the underlying disease. A detailed review of treatment methods which target the underlying disease is provided elsewhere.[69] In recent years, small-molecule compounds with the ability to reduce the formation, deposition and accumulation of Aβ amyloid aggregates have emerged as a promising method of treatment of AD. In vitro screenings led to the discovery of a large number of small molecules that may affect the amyloid aggregation pathway.[70] Some of these inhibited amyloid fibrils, whereas others arrested or reduced non-fibrillar

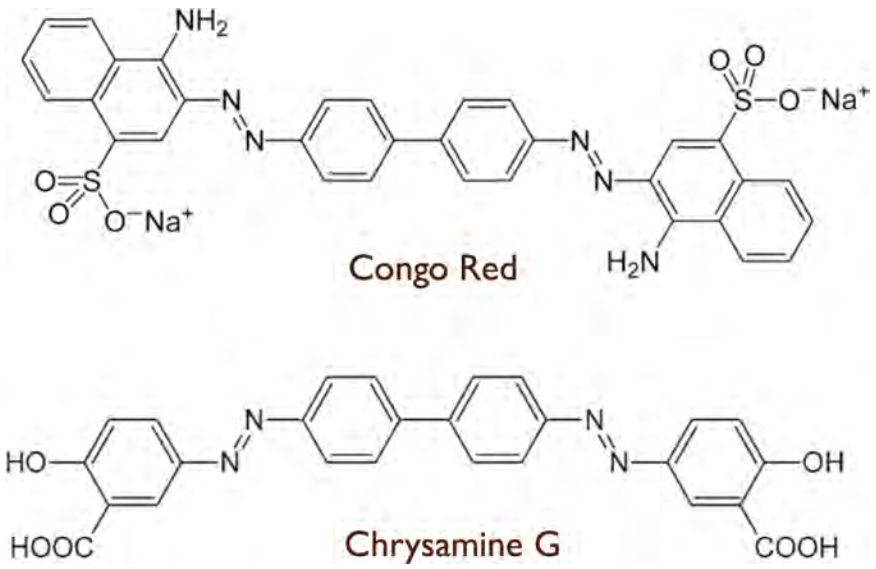

Figure 1.6: Chemical structure of amyloid-binding dyes Congo red and thioflavin T.

oligomer formation.[71,72]

A key pharmacological challenge of developing a drug for AD and other neurodegenerative diseases involves developing small molecules with the ability to penetrate the blood-brain barrier (BBB) in sufficient concentrations in the brain to achieve their therapeutic effects (ie. to inhibit amyloid formation). Although many small molecules appear to be effective in preventing amyloid formation in vitro, and attenuating their toxicity in cell cultures, many of them display poor BBB penetration and high toxicity to be immediately useful as therapeutics. Below we provide an overview of small-molecule inhibitors that are in vitro and in vivo inhibitors of amyloid fibrillation. Clinical trial data is mentioned if it is available.

1.4.1 Dye-based molecules

Among the first compounds discovered to bind amyloid fibrils were the dyes molecules used to identify their presence (Figure 1.6). Congo red (CR) was initially used in the histological detection of amyloid binding, where upon binding with CR, fibrils exhibit red-green birefringence when viewed with polarized light.[73] The binding affinity of CR

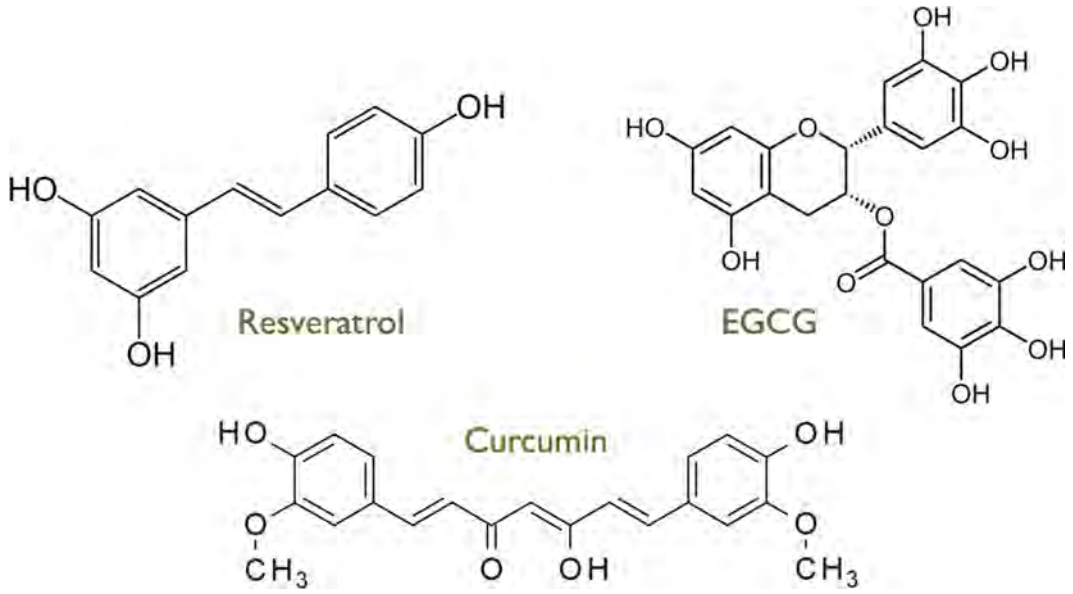

Figure 1.7: Chemical structures of flavonoids curcumin, EGCG and resveratrol.

with various fibrils is in the range of 0.1 - 1.5 μM.[74–76]

Early amyloid detection using CR revealed that CR not only binds to fibrils, but can also affect the amyloid aggregation pathway by interacting with one or more amyloidogenic species.[77] Fibril formation of amyloidogenic proteins Aβ,[78] prion,[79] and immunoglobulin light chain variable domain (SMA)[80] were found to be promoted by the presence of CR at low molar ratios, and inhibited at high molar ratios.

Although CR exhibits anti-amyloidogenic and anti-prion properties, its carcinogenic properties makes it a poor therapeutic candidate. Therefore, efforts were applied to find CR-based analogues, which maintain their anti-fibrillar aggregation activities, but improved the toxicity profile and BBB bioavailability. Chrysamine G (CG) is one such analogue of CR. CG has higher lipophilicity and lowered toxicity, and is capable of inhibiting aggregation and amyloidogenic toxicity in vitro and in vivo.[81–84]

1.4.2 Polyphenols

Polyphenols form a class of molecules found naturally in plants, and are composed of one or more aromatic phenolic rings, with multiple hydroxyl groups around these rings. Because of their antioxidant properties, the consumption of polyphenols have been reported to be beneficial for health. For example, resveratrol, a polyphenol found in red wine and -epigallocatechin-3-gallate (EGCG), a major phenolic component of green tea, were found to have cancer-preventative properties.[85,86] Currently, several clinical trials which examine their efficacy in cancer treatments are underway.[85,86] In recent years, polyphenols have gained additional attention due to their potential for treating AD.[87] Here we provide an overview of the compounds resveratrol, EGCG and curcumin, which have been well-characterized for their ability to inhibit amyloid formation.

In vitro, resveratrol inhibits the fibril formation of Aβ and the islet amyloid polypeptide (involved in type II diabetes), and attenuates amyloid-induced cellular toxicity.[88,89] Similarly, EGCG molecules promote self-assembly of amyloidogenic peptides Aβ[90] and α-synuclein[91] into "off-pathway" oligomers and inhibit the formation of mature fibrils by directly binding to monomeric forms of these peptides. Cell culture experiments indicate that micromolar concentrations of EGCG are protective against Aβ-induced cell death.[92,93] Curcumin, the main constituent of the spice turmeric, was reported to inhibit Aβ aggregation with IC_{50} values between 0.1-1 μM.[94–97]

Because of their anti-amyloidogenic activity, low toxicity, and ability to permeate across the BBB, polyphenols display therapeutic potential for the treatment of AD and related neurodegenerative diseases. Although it is well-known that ECGC, curcumin and resveratrol inhibit amyloid aggregation in vitro, their mechanism of action is still unknown. A key disadvantage of these polyphenol molecules is their high metabolic activity in the gastrointestinal system, which leads to poor absorption when administered orally.[85,98,99] Clinical trials to measure their efficacy in the treatment of AD are currently underway.

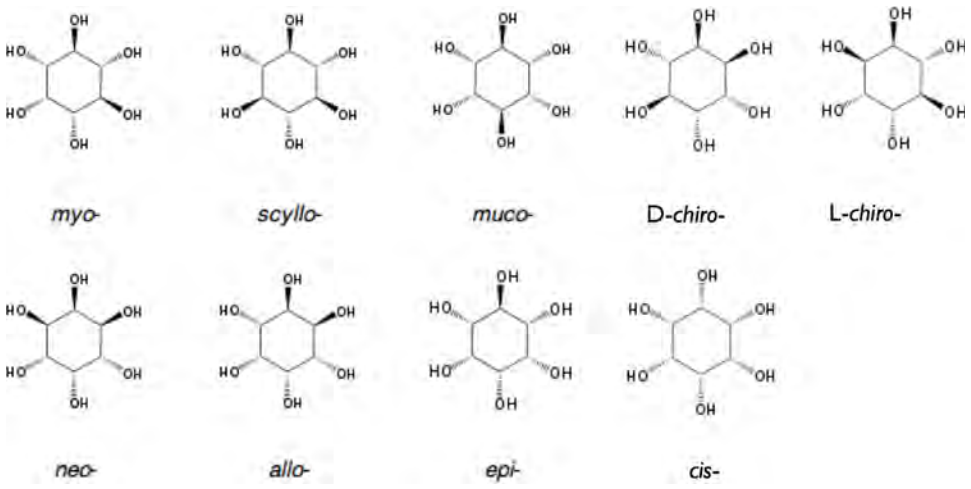

Figure 1.8: The nine stereoisomers of inositol.

1.4.3 Inositol

Inositol which has the molecular formula of $C_6H_{12}O_6$, is a class of simple polyols with nine naturally occurring stereoisomers (Figure 1.8). Out of these nine isomers, seven are optically inactive, and the remaining two (L- and D-*chiro*-inositol) are chiral enantiomers (Figure 1.8). *myo*-Inositol, the most abundant isomer, is ubiquitous in all eukaryotes and is a physiologically important osmolyte. Furthermore, *myo*-inositol is a precursor for inositol lipid synthesis: it is a constituent of phosphatidylinositol, an important phospholipid in membranes and second messenger systems. Once phosphorylated, *myo*-inositol phosphatides act as second messengers in intracellular signal transduction pathways.[100]

Inositol is found in high concentrations in tissues of the human central nervous system (CNS): *myo*- and *scyllo*-inositol have approximate concentrations of 5 and 0.1 - 0.5 mM in the CNS, respectively.[100] Accordingly, inositols also function as osmolytes in the CNS, where alterations in their concentrations are known to be associated with neuropathological conditions.[100,101]

In recent years, *scyllo*-inositol have been identified as a promising therapeutic candidate for the treatment of Alzheimer's Disease. Inositol was discovered as a possible amyloid inhibitor in a study where lipid bilayers composed of acidic phospholipids were found

to induce β structure in monomeric Aβ42.[102] Upon closer examination, *myo*-inositol, the headgroup of phosphatidylinositol, was found to be responsible for the induction of non-fibrillar β-sheet structure.[103,104] This finding led to in vitro studies which demonstrated stereochemistry-specific effects of inositol on Aβ fibril inhibition and cytotoxicity: *scyllo*-, *myo*-, and *epi*-, but not *chiro*-inositol inhibit Aβ42 fibril assembly, stabilize an oligomeric complex of Aβ42, and attenuate Aβ-oligomer-induced neurotoxicity in vitro.[105] Studies with a transgenic mouse model of AD demonstrated that the decrease in their AD-like symptoms after *scyllo*-inositol treatment was correlated with a decrease in the levels of soluble Aβ oligomers, suggesting that its beneficial effects are attributed to a reduction in the amount of high-order Aβ oligomers.[106]

Presently, *scyllo*-inositol has completed both phase I and II of human clinical trials for the treatment of AD.[107] In phase I trials, based on indicators such as brain plasma and CSF concentration, *scyllo*-inositol was found to be non-toxic to healthy individuals at concentrations effective for amyloid inhibition. From 2007 to 2011, phase II studies of *scyllo*-inositol were conducted with three dosage groups, where subjects in each group (comprised of 84 to 91 people) were administered 250 mg, 1000 mg or 2000 mg of *scyllo*-inositol (ELN005) orally twice a day. However, because of greater incidences of serious adverse events at the two higher dosages, only the lower dose was continued. Due to the decrease in the statistical power of the study after the removal of the high dosage groups, the efficacy of *scyllo*-inositol was not conclusive.[107] Taken together, these results suggest that *scyllo*-inositol may be a promising therapeutic for the treatment of AD, but that further improvements to enhance their therapeutic properties are most likely needed.[108,109]

1.5 Protein-ligand binding equilibria

Binding kinetics is concerned with the rate constant of ligand association (k_{on}) and dissociation (k_{off}). The ratio of the dissociation to the association rate constants establishes the equilibrium dissociation metric of the ligand ($K_d = k_{off}/k_{on}$), which determines the fraction of receptor occupancy at specific ligand concentrations. For the binding reaction given by

$$[Protein \cdot Ligand] \rightleftharpoons [Protein] + [Ligand] \tag{1.1}$$

The associated equilibrium constant (or the dissociation constant) of the above reaction is,

$$K_d = \frac{[Protein]\,[Ligand]}{[Protein \cdot Ligand]}, \tag{1.2}$$

K_d is a measurement of the affinity of a ligand for its binding site on the host protein with a unit of concentration. Pharmacologically, it is interpreted as the concentration of ligand at which 50% of the drug is bound to the protein. K_d is often used as quantitative indication of drug potency when screening for potential lead compounds. ΔG, the binding free energy of a ligand (with its receptor) is directly related to its K_d by,

$$\Delta G = -RT \ln K_d. \tag{1.3}$$

Hence, a small value of K_d indicates that the ligand is tightly bound (i.e. it possesses high affinity binding) to its binding site on the protein, and inversely, a high value indicates weak binding. Enzymes and their putative inhibitors typically have high binding affinities (or inhibition constants) in the nano to micromolar range. For example, the NSAID ibuprofen, which inhibits the enzyme COX-2, has K_d of $\sim$10 μM.[110]

In some cases, a ligand may be capable of interacting with its receptor at multiple binding sites. Such ligands may possess weak interactions with several receptor sites,

but are able to achieve binding specificity by forming many such weak interactions. An important example of this type of binding mechanism occurs in carbohydrate-protein binding, where multivalency plays an important role in their recognition. To characterize this type of interaction, a binding avidity constant that accounts for all possible binding modes of a ligand with its receptor is used.[111]

1.6 Intermolecular forces in biomolecular interactions

Protein-ligand interactions predominantly involve an array of non-covalent interactions. Van Der Waals forces are a weak intermolecular forces existing between molecules, which can be either attractive or repulsive. In particular, London dispersion forces is an weak attractive force between all molecules, which arises from the instantaneous dipole moment induced by electronic motions of nearby molecules. These forces predominantly account for the attraction between nonpolar molecules. Electrostatic effects also play an essential role in biomolecular recognition and structural stability. Fundamental to biomolecular interactions are hydrogen bonding and charge-charge interactions.[112]

A hydrogen bond is formed from electrostatic interactions arising between two dipoles: an acceptor and a donor. The acceptor group is comprised of an electronegative heavy atom (e.g. F, Cl, O), with exposed lone pairs of electrons, and the donor group is an electropositive atom. Furthermore, unlike dispersion forces, hydrogen bonds are directional, where the orientation of the hydrogen atom on the donor group is important to its formation. Hydrogen bonds formed between peptide groups involve N-H and O=C groups as the donor and acceptors, respectively. In intermolecular hydrogens formed in water, the O-H group acts as both the donor and acceptor group.

It has been estimated that the dissociation energies of hydrogen bonds range from weak (0.2 kcal/mol) to strong (40 kcal/mol).[113] A single water-water hydrogen bond is estimated to have an dissociation energy of approximately -3.2 kcal/mol.[113] The N-H $\cdots$

C=O hydrogen bond is estimated to be 4 kcal/mol in the gas phase,[114] and about -0.5 to -1.5 kcal/mol in solution.[115] **However, the binding free energy contribution of hydrogen bonding involved in the binding of a ligand and in protein folding is difficult to measure because the energetics of hydrogen bonds are sensitivity to the environment in which they are formed.**[114]

Notably, hydrogen bonds are ubiquitous in water, a medium essential to living organisms. A single water molecule is able to tetrahedrally coordinate four other water molecules to form a hydrogen bonding network. This hydrogen-bonded network of water molecules is known to impart to water its uniquely high heat capacity. Furthermore, hydrogen bonding plays a key role in stabilizing structures of several important biomolecules. In the double-helix structure of DNA, the complementary base pairing of DNA strands is held together via hydrogen bonding.

In proteins, intra- and intermolecular hydrogen bonds can be formed between the peptidic backbone, and polar or charged amino acid side chains of polypeptides. These hydrogen bonds define protein secondary structure (helices and β-sheets), and impart stability to their overall tertiary structures. For example, hydrogen bonding patterns determine the arrangement of peptide strands into either parallel or anti-parallel β-sheets. In addition, peptides with their peptidic backbone hydrogen-bonded to each other to form in-register β-sheets are at the core of the cross-β structure of amyloid fibrils (see Section 1.1).

Salt bridges are considered to be the strongest type of hydrogen bonds in biomolecules. In proteins, salt bridges are a type of hydrogen bond formed between two oppositely-charged amino acid side chains (e.g. arginine and aspartic acid), and are thought to contribute to the overall stability of the protein.[112,116]

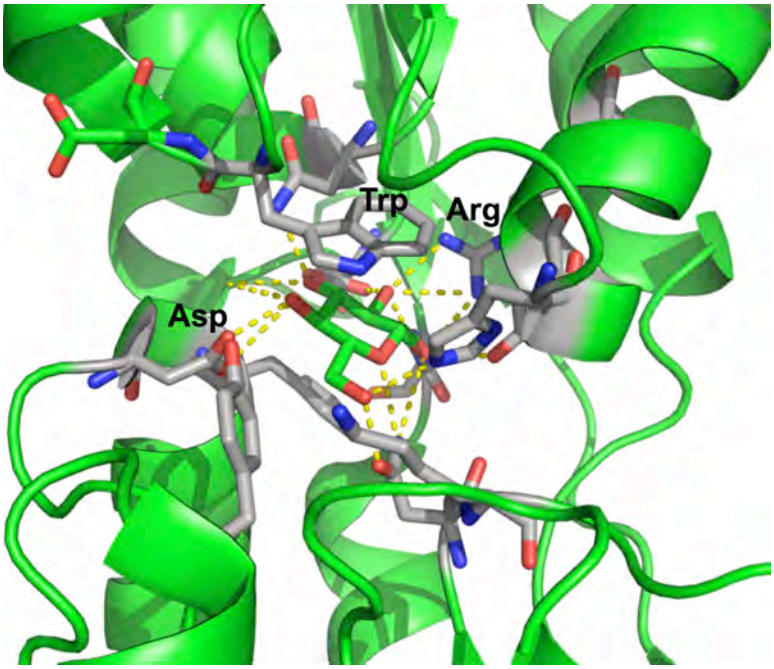

Figure 1.9: The structure depicts glucose bound to a galactose chemoreceptor protein (Ref: Vyas, N. et al, 1991; Rendered from PDB ID: 2GP). Hydrogen bonds between the protein and glucose are depicted in dotted yellow lines.

1.7 Protein-carbohydrate interactions

Carbohydrates are the most abundant organic compounds in nature and are an important component of living cells. They are sources of energy, building blocks, and recognition elements for both plants and animals. Proteins capable of binding carbohydrates mediate processes such as antigen recognition by the host immune system,[117,118] signal transduction,[119] bacterial infections,[120] and cell-cell adhesion.[121] Because protein-carbohydrate interactions are involved in many fundamental cellular processes, a great deal of research in the past three decades has been devoted to understanding the molecular basis of their binding mechanism.

Monosaccharides, or simple sugars, are carbohydrates that cannot be hydrolyzed into simpler compounds, whereas polysaccharides are carbohydrates that can be hydrolyzed to form many monosaccharide units. Polysaccharides are naturally occurring polymers of carbohydrates. Smaller polysaccharides of about three to ten units long are sometimes

called oligosaccharides. Furthermore, sugars are chiral compounds: a sugar molecule can either be in the D or L configuration. Most naturally occurring sugars are of the D series.

In the past 20 years, the structures of many sugar-binding proteins (or lectins) have been resolved by X-ray crystallography.[122] Lectins may have monosaccharides or polysaccharides as substrates. An example of a bound glucose in the active site of its protein receptor is depicted in Figure 1.9. Carbohydrate-protein interactions constitute a complex subject. Our discussion here will be focused on the general features of binding sites and modes of monosaccharides.

Protein-carbohydrate interactions are relatively weak compared to certain protein-protein interactions. For example, dissociation constants for most lectin-monosaccharide interactions are in the millimolar range, implying that lectins display low binding specificity for their ligands. Furthermore, although the affinity increases with oligosaccharides, the dissociation constants are still in the micromolar range. However, the ability of carbohydrates to form multivalent interactions with their receptors, in part, helps lectins achieve their binding specificity. This mechanism is more generally known as the avidity effect and has been well-documented for several protein-carbohydrate systems.[122]

Sugar binding sites are distinct from other ligand binding sites in that they mostly occur at the surface of proteins, and form cavities or grooves. Although any residue may be involved in binding carbohydrates, the most frequently occurring residues are aromatic (Trp, Tyr and Phe), polar and charged residues (Glu and Asp).[123] Carbohydrate binding sites have been generally classified into two groups: deep, solvent-inaccessible binding pockets and shallow grooves, displaying high and low sugar-binding affinities, respectively.[122,123]

Atomic features of protein-carbohydrate interactions are generally characterized by the formation of hydrogen bonds and nonpolar interactions with aromatic moieties.[123] Often, a delicate balance between hydrophobic and hydrogen bonding interactions is required for in protein-carbohydrate binding, where sometimes a slight change in the

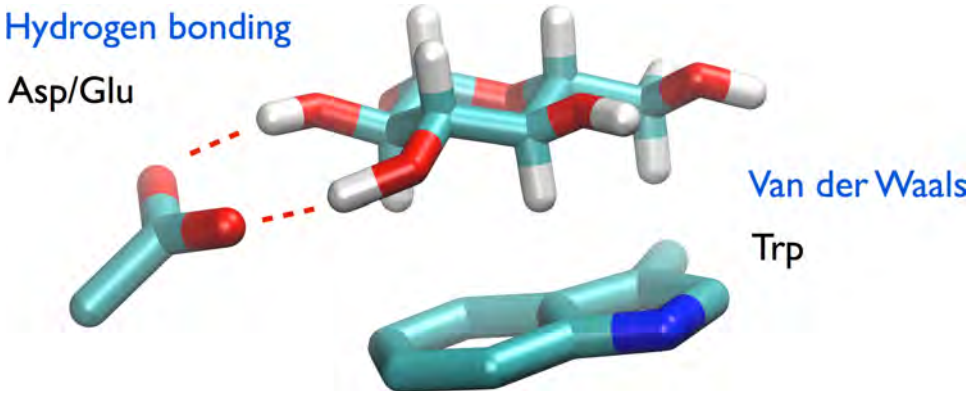

Figure 1.10: A schematic of a monosaccharide simultaneously forming Van der Waals interactions with an aromatic moiety (Trp) and bidentate hydrogen bonds with carboxylate group of an acidic residue.

stereochemistry of the carbohydrate substrate can alter its binding specificity.[124]

Because sugars have numerous hydroxyl groups (-OH), hydrogen bonds are ubiquitous in sugar-protein interactions. The hydroxyl groups can interact with both polar and charge groups of amino acids, and the peptidic backbone. A monosaccharide is often found to form planar bidentate hydrogen bonds with the carboxylate groups of aspartic and glutamic acids (Figure 1.10).

Another characteristic binding mode is the nonpolar stacking between hydrophobic faces of the sugar ring and the side chains of aromatic residues (Figure 1.10). This stacking mode, often referred to as CH-π interactions, is thought to arise from the entropically favorable packing of the hydrophobic faces of sugar with aromatic rings, and the enthalpically favorable CH-π orbital interactions.[125]

1.8 Structure-based rational drug design

Rational drug design is often applied to optimize ligand binding specificity in an effort to increase the efficacy of the putative drug, and decrease adverse side effects (toxicity) in the human body. Structure-based rational drug design is a drug discovery paradigm that has yielded success in the discovery of drug candidates. A target is first identified and

its role in the relevant disease pathway characterized. Then, the molecular structure of the target protein is determined using techniques such as NMR or X-ray crystallography. By having the molecular structure of target proteins, putative enzymatic active sites or binding sites may be identified. If the structure of the target is determined in its bound state, protein-ligand interactions can be characterized at the molecular-level. At this stage of the pipeline, high-throughput screening using a chemical compound library can be performed to find compounds (e.g. inhibitors) with high binding affinities (low K_d) to the binding site. In vitro experiments may be conducted to assess the structure-activity relationship of these compounds. The information obtained from these experiments can either feedback into the design cycle to find better inhibitors, or be used to guide in vivo experiments.

1.9 Molecular dynamics simulations

In general, MD simulations are a useful tool to study the structure, dynamics, and interaction of biomolecules (see Chapter 2 for methodological details). MD simulation studies have been useful in studying many existing fundamental problems of biology and biochemistry, including protein folding, biomolecular self-aggregation, and protein-ligand binding. MD simulations employ an empirical mathematical function to describe the atomic interactions in a molecular system, and together with classical laws of Newtonian mechanics, atomic trajectories of motion are generated. Thermodynamic and kinetic properties can then be extracted as time averages from these trajectories and used to make a number of predictions that are often experimentally challenging to observe or measure.

With increasingly faster and cheaper computer hardware, structure-based computer modelling and simulations of protein-ligand interactions are becoming a key component of the modern drug discovery process. For example, computer simulation techniques

such as molecular dynamics (MD) simulations may be used to predict protein-ligand binding free energies, a quantity often used to evaluate how well a ligand binds in a high-throughput screening.

Alternative in silico methods such as computational docking, where the energetics of binding is typically estimated without accounting for either ligand or protein flexibility may be used to obtain a crude estimate of the binding affinity. Although docking is fast, its inaccuracy often leads to many false positives. Current state-of-the-art computational binding studies use molecular dynamics (MD) simulations, which is a much more accurate, but computationally expensive method to predict binding free energies. In MD simulations, the protein and its putative ligand is allowed to relax and freely move about in the system, allowing a realistic estimate of the binding free energy to be made. Simulation trajectories of protein-ligand binding can be used to quantitatively assess whether a chemical change to a compound will produce a more potent drug candidate (e.g. residues may be "mutated" in silico).

1.9.1 Challenges and limitations of MD simulations

For molecular simulations to reliably predict and guide experiments, they need to be sufficiently accurate, possess a correct representation of the experimental conditions, and adequately sample the relevant biomolecular motions.[126] Ideally, a simulation should be at least 10 times longer than the slowest important timescale in a system.[127] However, sampling relevant events on this timescale are intractable for many important biologically-relevant phenomena (eg. protein folding, amyloid formation, ligand binding and conformational isomerization) because they occur on timescales (greater than 1 ms) typically not reachable via MD simulations using current available computing resources. Although modern simulations studies routinely approach microseconds in sampling, only a few studies to date were able to reach timescales of milliseconds.[128–130]

Consequently, running a single continuous molecular dynamics (MD) simulation alone

is unlikely to achieve sufficient sampling of the important states of many biologically relevant systems. For this reason, computational algorithms which enhance sampling of the energy landscape of biomolecular systems are often employed to overcome some of the limitations of conventional MD simulations.

1.10 Recent progress in elucidating the molecular mechanism of small-molecule binders and inhibitors of amyloidogenic species

Although the structure-based rational drug discovery paradigm is well-suited to finding inhibitors of globular proteins such as enzymes, designing small-molecules which may inhibit amyloid formation present additional challenges. Amyloid fibrillation is a multi-stage process involving different species at each stage. Due to the heterogeneous and non-crystalline nature of prefibrillar species, experimental determination of the molecular structures of these amyloid species remains a challenge. Further compounding experimental challenges, these small molecules can interact with amyloidogenic species at different stages of aggregation.

Moreover, the binding mechanism of small-molecule inhibitors of amyloid aggregation is not described completely by the classical enzyme-inhibition model. An inhibitor concentration in the micromolar to millimolar range is often required to observe amyloid inhibition, suggesting that these small-molecule inhibitors are non-specific binders. By contrast, substrates of enzymes typically exhibit high binding specificity with binding affinities in the nanomolar range. Taken together, the above challenges have significantly impeded the determination of the molecular basis of amyloid inhibition by small-molecule inhibitors using existing experimental methods.

By contrast, computer simulations are not limited by these experimental challenges

and can provide the atomistic level of detail needed to elucidate the action of small-molecules on the inhibition of amyloid formation. As a result, MD simulations have played a key role in advancing the understanding of the binding mechanism of these small-molecule inhibitors. However, elucidating the molecular basis of these small-molecule inhibitors poses a number of difficulties for simulation studies. The structural disorder of amyloid-forming peptides impedes obtaining statistically-meaning properties from MD simulations. Furthermore, because it is not known whether small-molecule inhibitors may interact with amyloidogenic monomers or aggregates, it is often necessary to examining their binding with several amyloidogenic species in order to gain a complete understanding of their mechanism of action.

In recent years, a large number of MD simulation studies were published between the years of 2007 to 2013 (the time of writing of this thesis). Below I will provide a review of the recent progress in elucidating the binding mechanism of small molecule inhibitors of amyloid formation. While the central focus of the review is on MD simulation studies, experimental results are discussed if they are available.

1.11 Thesis objectives and organization

Alzheimer's Disease (AD) is a progressive neurodegenerative disease characterized pathologically by the presence of extracellular fibrillar deposits of β-amyloid (Aβ), a 38 to 42 residue protein that is produced normally as part of the cellular metabolism. Scyllo-inositol is a promising potential therapeutic compound for AD treatment, which is currently in phase two of clinical trials. Both scyllo-inositol and one of its stereoisomers, epi-inositol, have been shown to effectively block the accumulation of Aβ oligomeric assemblies and reduce AD-like symptoms in a transgenic mouse model of AD. Furthermore, in vitro, scyllo-inositol and its stereoisomers myo-inositol, epi-inositol, and chiro-inositol, have been demonstrated to stabilize Aβ42 oligomers, disassemble preformed Aβ42 fibrils,

and reduce Aβ42-induced neurotoxicity in a stereochemistry-dependent manner. However, the specific molecular interactions of inositol and its effect on Aβ aggregation at the molecular level are not known. At present, experimental approaches lack the ability to determine the precise mechanistic mode(s) of action of inositol as the molecular structures of various intermediates in the amyloid aggregation pathway are not known. Moreover, observed intermediates morphologically distinct from fibrils are experimentally very difficult to detect and isolate.

The primary objective of my research is to use molecular dynamics (MD) simulations to elucidate the molecular basis for the activity of inositol by determining its effect on the structure, thermodynamics, and kinetics of Aβ aggregation. Our central hypothesis is that inositol acts by binding to one or more of the aggregated form of Aβ. Determining the effect of inositol binding on the full-length Aβ protein is computationally challenging and requires the use of many cores on high performance computing systems. The formation of amyloid follows a complex aggregation pathway, where different intermediate Aβ aggregate species have been implicated in the disease. Therefore, a meaningful study requires examining inositol binding with different self-aggregated peptide states, from monomeric to fibrillar aggregates.

To this end, I have carried out systematic comparative studies of amyloidogenic peptides and their aggregates of increasing sequence and composition complexity and characterized the respective role of specific interactions of inositol with the backbone and sidechains (Figure 1.11).

Inositol molecules were shown to have stereochemistry-dependent activity in vitro and in vivo. Chiro-inositol is a stereoisomer that was shown to be inactive in inhibiting amyloid formation. Thus, in all of my studies, I have comparatively examined chiro- and scyllo-inositol with each of the aggregates in the pathway to determine the stereochemical basis of the activity of inositol. In addition, we also perform control simulations in the absence of inositol. In order to achieve the sampling necessary to obtain meaningful

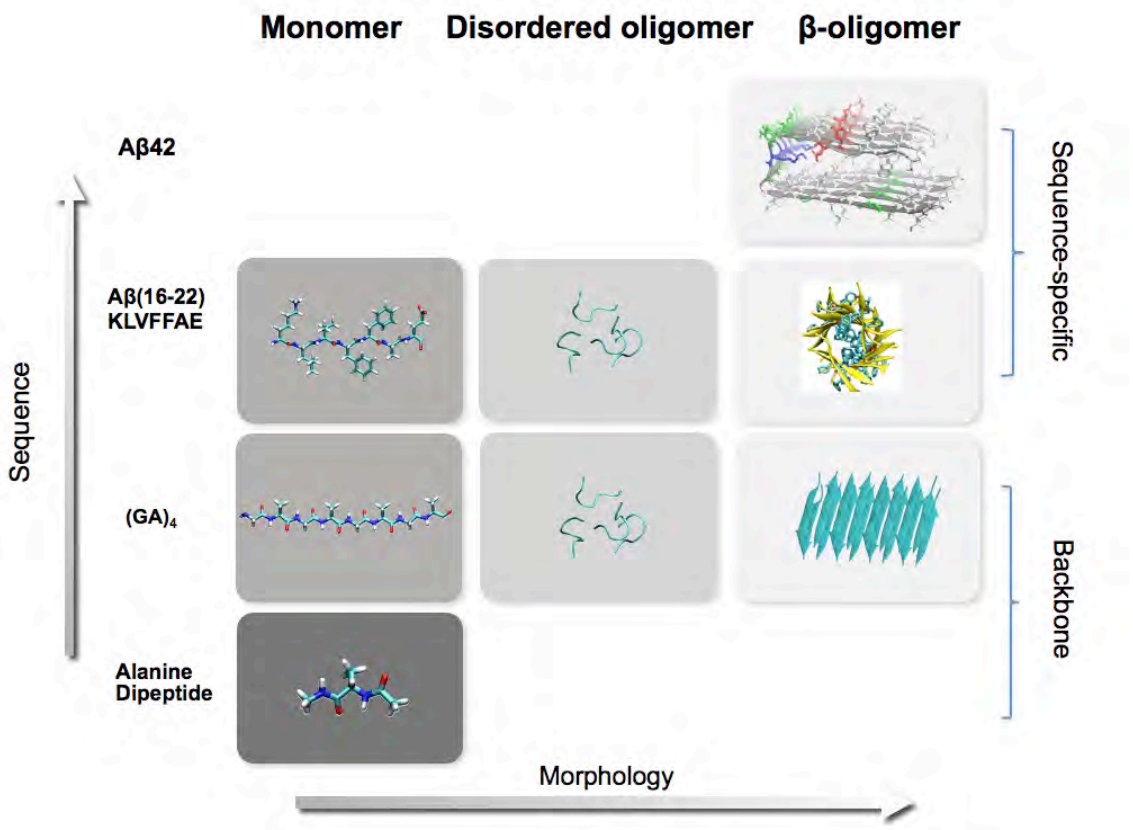

Figure 1.11: Using a reductionist approach, we progress from examining model amyloidogenic peptides and aggregates to more complex systems involving the full-length Aβ42 peptide.

statistics from our simulations, we perform thousands of independent MD simulations of scyllo- and chiro-inositol with (1) single peptides; (2) small aggregates; (3) large ordered aggregates of various amyloidogenic sequences (Figure 1.11).

I first systematically examined the binding modes of inositol with simple models of amyloidogenic peptides (Chapter 3).

Next, I characterized the binding of inositol with the peptide KLVFFAE or Aβ(16-22), a short fibril-forming fragment important for initiating amyloid formation in the full-length protein (Chapter 4).

Based on my results from Chapters 3 and 4, which indicated that inositol acts on protofibrils of Aβ, rather than monomeric or non-beta-sheet oligomeric states, in Chapter 5, the binding of inositol was exclusively examined with the protofibril of full-length Aβ42.

Finally, in Chapter 6, I demonstrate the general applicability of the methodology developed in this thesis by applying similar MD simulation approaches to examine protein - carbohydrate binding.

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