Protein Stability in Shear Flow

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Declaration

This is to certify that

- the thesis comprises only my original work towards the PhD,
- due acknowledgement has been made in the text to all other materials used,
- the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

____________________________________

Innocent Berbelle Bekard
Abstract

Given that globular proteins show a strong conformation-function relationship, the stability of a native protein structure is essential for its function. Aberrant proteins, resulting from structural instabilities in native protein conformations, and consequent aggregation, evolve a gain-of-function pathogenesis which has serious implications in industry and medicine. Therefore, it is important to appreciate the key factors that perturb the solution conformation of protein systems leading to aggregation. Considering the fact that proteins generally function in solution form, and those solutions have an inherent tendency to flow, knowledge of the stability of protein solutions in shear flow is essential. This thesis employs a combination of spectroscopic and microscopic techniques to study the conformational dynamics and morphological transformations of bulk peptide/polypeptide solutions in both uniform and heterogeneous velocity gradients. Preliminary studies in this thesis demonstrate that protein denaturation and subsequent aggregation can be probed using intrinsic protein fluorescence. The induction of protein aggregation was found to be greatly enhanced in heterogeneous flow regimes. Studies in a well defined flow field, Couette flow, revealed that the hydrodynamic stress generated in such flow regimes induce the unfolding of the helical segments of natively folded insulin; a prerequisite for aggregation and amyloid fibril formation. Further analysis of the shear-effect on α-helical conformations was performed using the homopolypeptide poly-L-lysine as a model protein system. The results reveal that the shear-induced unfolding of α-helical segments depends on both the shear rate and the duration of its application. An assessment of the chain-length-dependence of this phenomenon revealed that, contrary to classical theory, the strain in a given flow field varies inversely with the chain-length of α-helical poly-L-lysine. Collectively, the results provide new insight into existing theories in polymer physics. More importantly, it provides quantitative information on the conformational dynamics of peptide/polypeptide solutions in shear flow. This report is relevant to quality control measures during the commercial isolation and purification of protein products, and might help explain the role of shear stress, originating from pulsatile blood flow, in protein misfolding diseases and vascular disorders.
To my mother, Theodora Gyinokang.
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Walking down this path to knowledge has brought its fair share of moments of anxiety, excitement and frustration. It is interesting to note that one can experience all three on the same day when an experimental setup looks great at first light, proceeds as expected by noon, and takes a nose dive for the worst by sunset. But for the unyielding support of many, I may not have made it down this path to knowledge in one piece.

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1. Introduction

Proteins are linear polymer chains of combinations of the twenty amino acid residues (1). These complex macromolecules form an essential part of an array of biological processes in living systems, such as structural components of cells, biological catalysts (enzymes) and chemical messengers (hormones) (2). The functional properties of proteins depend on their unique three-dimensional structure which is determined primarily by the amino acid sequence of the polypeptide chain (1, 2). The energy threshold between the native and denatured state of a protein is low, thus the physicochemical environment is an equally critical determinant of protein structure and function (3).

Four categories of protein structure namely primary, secondary, tertiary and quaternary structure are recognized (1). Primary structure is defined by the linear sequence of amino acid residues in the main chain. Secondary structure describes regions of periodic conformations, such as α-helices and β-sheets, of the polypeptide chain. The tertiary and quaternary structures are defined by the form of the fully folded polypeptide chain, and the association of two or more polypeptide chains into a multi-subunit structure respectively.

The unique solution conformation of folded proteins arise from their component secondary structures; the most recurrent being α-helix and β-sheet (2). The occurrence of these secondary conformations in folded proteins was first proposed by Linus Pauling, Robert Corey, and Herman Branson in 1951 (4). The predominance of any particular secondary conformation in the overall protein structure is determined by the propensity of the amino acid residues of the polypeptide chain to adopt that conformation.

In aqueous solution, hydrophobic amino acid residues are concealed in the protein matrix whereas residues with polar side chains makeup the solvent-exposed segments in contact with the surrounding medium. The stability of the native protein structure originates from a network of intra-molecular hydrogen bonds and hydrophobic interactions between amino acid residues and segments of the polypeptide chain respectively (2, 5). For
example, the α-helix conformation is stabilized by hydrogen bonds between the amide (NH) and carboxylic (CO) groups of the main chain (2).

It has long been recognized that perturbation of the native structure of globular proteins often leads to the evolution of dysfunctional, aggregation prone species (6-9). This phenomenon informed the notion that proteins show a strong conformation-function relationship. Proteins trapped in such metastable conformations, irrespective of their primary structure, precipitate out of solution as amorphous aggregates and/or amyloid fibrils rich in β-sheet (10, 11). Protein aggregates, especially amyloid fibrils, are recognized as the pathologic hallmark of protein conformational disorders (e.g. Alzheimer’s disease)(12) and vascular disorders (e.g. atherosclerosis) (13). Furthermore, the evolution of such dysfunctional protein aggregates during the commercial isolation and purification of protein products is an essential quality control issue confronting the biotechnology industry (14). Given the biological significance of protein aggregation, unravelling the molecular basis for protein folding and misfolding has been at the forefront of research particularly in the biosciences. While some insight has been gained over the years, a complete understanding of this phenomenon remains elusive.

A common experimental approach to studying protein denaturation, in natively folded proteins, involves the disruption of the native protein conformation (unfolding) via the manipulation of solvent conditions. These conditions include temperature, pH, ionic strength, and the introduction of chemical denaturants (e.g. guanidine and urea) (15). Refolding of the unfolded polypeptide chain is probed by the reversal of these conditions. A similar approach is applied to investigate protein aggregation and amyloid fibril formation (15). Here, protein samples are continuously exposed to a destabilizing environment in order to promote aggregation.

Structural techniques commonly applied in these studies include fluorescence spectroscopy, dynamic light scattering and circular dichroism spectropolarimetry. These are complemented by imaging techniques such as electron microscopy (EM) and atomic force microscopy (AFM) to characterize the morphology of protein aggregates. In
fluorescence studies of protein denaturation, the hydrophobic dye 1-anilinonaphthalene-8-sulfonate (ANS) is often employed to probe solvent-exposed hydrophobic groups during the unfolding of natively folded proteins (16, 17); a prerequisite for aggregation and amyloid fibril formation. The association of solvent-exposed hydrophobic groups into prefibrillar aggregates is probed by light scattering techniques (18, 19), and their polymerization into fibrillar forms probed by the amyloid dye Thioflavin T (20, 21). Conformational transitions between the secondary structural elements of the polypeptide chain during protein aggregation are often investigated using circular dichroism spectroscopy (22, 23). While technological advancement in optical techniques over the years has significantly improved our understanding of protein denaturation, as stated earlier, the molecular mechanism underlying this phenomenon remains to be fully understood. It is anticipated that an in-depth knowledge of the key steps and contributors to protein aggregation would be crucial for the development of therapeutic interventions to counter and/or prevent the debilitating effects of neurodegenerative diseases. In addition, such information will inform the design of quality control systems in industry to ensure the stability of the physical and chemical integrity of protein therapeutics during processing, transportation and storage.

A substantial part of the work reported herein is dedicated to understanding the influence of fluid forces in protein denaturation and aggregation. It is noteworthy that this parameter is only one of the myriad of factors associated with protein denaturation and amyloid fibril formation (15). However, given that proteins are usually functional in solution form, coupled with the fact that solutions have an inherent tendency to flow past a solid boundary, potentially incurring a shear stress, it is essential to investigate the shear-stability of proteins in flow. In fact the conformational dynamics of biopolymers in shear flow has received considerable attention in the literature since the late sixties (6). This is mainly because knowledge gained is of immediate relevance to both industry and physiology.

However, the complexity associated with studying the structural dynamics of proteins in shear flow has lead to a significant number of theoretical studies, rather than
experimental investigations, in this field (24-26). Preliminary investigators were confronted by the lack of optical techniques with the sensitivity and time resolution to directly probe the influence of fluid forces on the solution conformation of proteins under shear flow. Hence, information gathered was based on indirect experimental evidence (6). More recently, novel fluorescence techniques have been employed to monitor the structural dynamics of proteins in shear flow, but are limited by the inability of this structural technique to measure and quantify the secondary structural components of the protein systems under study (27, 28). Consequently, the information gained from these studies is only qualitative in nature.

The main theme of the work described here is to present a novel experimental approach designed to directly monitor the structural and conformational dynamics of protein systems, \textit{in situ} and in real time, using circular dichroism spectropolarimetry. Circular dichroism is a simple, yet excellent, quantitative structural technique (22). This technique is complemented by fluorescence spectroscopy, another sensitive structural technique, and atomic force microscopy to probe the morphology of protein aggregates. Bovine insulin and amyloid-β are the sample protein systems used in these studies. Both systems are well characterized and have been found to form fibrillar aggregates under favourable solution conditions (29, 30). In addition, poly-L-lysine, a homopolypeptide which is widely used as a model protein system (22), was employed to further understand the conformational dynamics of biopolymers in simple shear flow. The data and information presented here sheds new light on the physics of these bio-macromolecules in shear flow. More importantly, it might help explain the role of haemodynamic drag in protein conformational disorders and vascular disorders in microcirculation.

In this thesis, chapter 2 describes the premise of the work presented herein by reviewing the existing literature on protein denaturation, aggregation and amyloid fibril formation and its implications in industry and medicine. Emphasis is placed on the influence of hydrodynamic shear stress on protein structure and function; the main subject of this thesis.
The experimental techniques applied in this work are described in chapter 3. These include fluorescence spectroscopy, circular dichroism and atomic force microscopy. A custom-built shear-cell setup used to study the dynamics of various protein and polypeptide systems in Couette flow is also described.

The first experimental section (chapter 4) describes a novel experimental approach, designed during the preliminary stages of this work, which exploits the intrinsic Tyr fluorescence of insulin to investigate the sequence of structural changes accompanying insulin aggregation. The relevance of obtaining such critical information from the protein itself is discussed. More importantly, the sensitivity of this technique was applied in subsequent studies on the conformational dynamics of bovine insulin in simple shear flow.

In chapter 5, the effect(s) of hydrodynamic shear stress, originating from a heterogeneous velocity gradient (stirring), on the aggregation and subsequent fibrillation of amyloid-β is presented. A molecular mechanism that describes this structural transition is also presented. It is noteworthy that senile plaques of amyloid-β serve as the pathologic indicator of Alzheimer’s disease (12).

In chapters 6 and 7, a controlled flow field (Couette flow) is applied to characterize the influence of hydrodynamic drag on the structure of protein and polypeptide systems. Studies of the shear-stability of the helical segments of bovine insulin in simple shear flow are presented in chapter 6. Here, fluorescence and circular dichroism spectroscopy were employed to characterize the structural dynamics of insulin under shear flow. The morphology of sheared insulin samples was investigated using atomic force microscopy.

In chapter 7, the shear-stability of different chain lengths of the homopolypeptide poly-L-lysine, initially in an alpha helix conformation, was examined using real-time circular dichroism measurements in situ.
The main outcomes of these studies, as well as considerations for the advancement of future investigations, are summarised in chapter 8.
2. Background & Literature Review

2.1 Protein folding, misfolding and aggregation

Newly synthesised polypeptide chains in living cells go through a folding process, assisted by internal regulatory systems such as molecular chaperones, to attain their functional native structure. Aberrant proteins, a result of production errors, inherited or acquired amino acid substitutions, or damage due to oxidative modifications *inter alia*, can in many cases misfold and get trapped in dysfunctional conformations. Under conditions that have yet to be fully understood, such misfolded proteins often assemble and deposit as amyloid plaques within the intra- or extra-cellular environment (8, 31). The possible structural configurations that can be accessed by newly synthesised polypeptide chains are shown in figure 2.1.

![Figure 2.1](image)

Figure 2.1: ‘A unified view of some of the types of structure that can be formed by polypeptide chains’ (8).

Several studies have shown that proteins possess an inherent propensity to misfold and aggregate into the highly ordered and thermodynamically stable amyloid fibril structure, under solution conditions that destabilize the native protein conformation but still allow
other molecular interactions (10, 32). It is argued that this may be true for the test tube situation, but *in vivo* it seems that amyloid fibril formation is restricted to only a few polypeptides (11). About twenty-five are known to-date. Whatever the case may be, the question arises as to why certain proteins misfold *in vivo* and deposit as amyloid fibrils although the cell has evolved its own protective mechanisms namely: (i) molecular chaperones, which screen exposed hydrophobic surfaces and ensure correct protein folding, and (ii) the ubiquitin-proteasome system, which degrades misfolded proteins hence preventing their aggregation (33).

It is believed that both soluble aggregates and fibrous deposits of dysfunctional proteins evolve a gain-of-function pathogenesis that is unique to protein misfolding diseases (33, 34). However, it is argued that fibrillar deposits, which result from the coalescence of misfolded proteins, may not be the neurotoxic species in protein misfolding diseases (35). It is proposed that amyloid fibrils may rather represent a non-functional state, albeit inseparable from the disease process, or even represent the presence of an inherent detoxifying mechanism that ‘entrap’ potentially injurious endogenous products into ‘quiescent’ aggregates (35). For this reason, the initial notion that the inhibition of fibril formation is an ideal therapeutic intervention to neurodegenerative diseases is debated.

### 2.2 Amyloid fibrils

The Nomenclature Committee of the International Society of Amyloidosis suggests that the term ‘amyloid’ is presently best restricted to *in vivo* material with typical staining reactions (e.g. with congo red) (11). Therefore, amyloid fibrils are defined as elongated, insoluble protein aggregates deposited *in vivo* in amyloid diseases.

However, several *in vitro* studies show that a variety of proteins and peptides can self-assemble into long, often twisted, insoluble aggregates with structural and biochemical characteristics similar to that of the amyloid fibrils observed *in vivo* (36). These are referred to as amyloid-like fibrils. The fibrils, so formed, adopt a β-sheet conformation which is composed of extended strands (β-strands) of the polypeptide chain, and stabilized by a network of interconnecting hydrogen bonds and hydrophobic interactions.
between the β-strands. In fact, synchrotron X-ray diffraction analysis of fibrillar aggregates from an array of polypeptides, which share neither sequence homology nor biological function, suggest that the fibrils show similar fibre diffraction patterns consistent with a helical configuration of β-sheets parallel to the fibril axis, with the component strands perpendicular to this axis (37). This further suggests that the unique characteristics of amyloid fibrils are independent of the precursor protein. Atomic force microscope images of fibrillar amyloid-like aggregates of insulin and amyloid-β are shown in figure 2.2.

![Figure 2.2: Amyloid-like fibrils of amyloid-β (A) and bovine insulin (B).](image)

### 2.3 Protein denaturation and aggregation probes

Detection of protein denaturation is essential for the reliable characterization of protein aggregates and investigation of the aggregation kinetics of amyloid fibrils (38). Advancements in the sensitivity of structural techniques, responsive to the conformational dynamics of polypeptide chains upon perturbation, have proven versatile in improving current knowledge on the molecular basis of protein folding/unfolding (23, 39). Fluorescence spectroscopy and circular dichroism spectropolarimetry are the most common structural techniques applied in these studies, and are discussed in detail in chapter 3. A major advantage here is that both techniques are relatively easy to use and do not require complex sample preparations.
Fluorescence techniques rely on intrinsic and/or extrinsic fluorescent probes for data acquisition, whereas circular dichroism exploits the solution configuration of the polypeptide backbone and/or aromatic amino acid side chains in a polypeptide chain.

In particular, extrinsic fluorescent probes have been instrumental in resolving the complex structural transitions involved in protein aggregation (40). Traditionally, amyloid fibrils are identified by their unique tinctorial features on interacting with the histological dyes thioflavin T (ThT) and congo red. However, the specificity of these extrinsic dyes has yet to be fully understood (41). Between the two dyes, ThT is preferred for in situ kinetic studies of amyloid fibril formation as congo red is believed to inhibit fibril assembly (42). For natively folded proteins, the hydrophobic dye ANS is commonly employed to detect partially folded intermediates believed to precede fibril formation. The molecular structures of ThT and ANS are shown in figure 2.3. It is noteworthy that the intrinsic fluorescence of proteins show sensitivity to conformational changes in the native structure and have been successfully applied as a complementary technique in protein denaturation studies (39).

![Figure 2.3: Molecular structures of (A) Thioflavin T and (B) ANS.](image)

Thioflavin T is a benzothiazole dye that exhibits enhanced fluorescence on interacting with the multimeric β-sheets of amyloid fibrils (20). A molecule of ThT consists of a pair of benzothiazole and benzaminic rings freely rotating around a shared C-C bond (21). Aqueous solutions of ThT have been found to show excitation and emission maxima at 342 nm and 430 nm respectively.
In the presence of fibrillar aggregates however, the spectral features of ThT are altered, showing excitation and emission maxima at 442 nm and 482 nm respectively (43, 44). In spite of the widespread use of ThT in investigations of the kinetics of amyloid fibril formation, its mode of binding to amyloid fibrils and the consequent changes in its spectral properties is ill understood. It is proposed that ThT micelles bind along the multimeric β-sheet cavities of fibrillar aggregates, and that the observed spectral changes is attributable to steric hindrance of the free ring rotation of individual ThT molecules (21).

The negatively charged hydrophobic dye ANS is commonly used for the early detection of intermediate states (e.g. molten globules) during protein denaturation and aggregation (16, 40). Its application in protein studies was pioneered by Stryer in 1965 (45). The spectral features of ANS show weak fluorescence in water, with an excitation and emission wavelength maxima of 350 nm and 521 nm respectively. However, upon association with solvent-exposed hydrophobic moieties of proteins (and other macromolecules), ANS displays an enhanced fluorescence emission, accompanied by a blue-shift in the emission wavelength maximum from 521 nm to 470 nm. Its ability to bind hydrophobic moieties of proteins and peptides is attributed primarily to electrostatic interactions, with contributions from hydrophobic forces and stoichiometric relationships (17). Hence, its mode of action is dependent on the amino acid composition of the polypeptide chain as well as solution conditions including pH, viscosity, polarity and temperature (17, 40).

2.4 Proposed mechanisms of protein aggregation
Several models have been proposed to explain the mechanism of amyloid fibril assembly. Jarrett and Lansbury(46) suggest that protein aggregation follows a nucleation-polymerisation mechanism, which explains the observed sigmoid curve reported in kinetic studies of amyloid assembly. A more popular model is the nucleation conformational conversion mechanism (NCC) (figure 2.4) proposed by Serio et al. (47). This model recognises the fact that protein aggregation is accompanied by a conformational transition of the disordered polypeptide chain into a more stable structure;
primarily β-sheet. By contrast, others propose a mechanism that involves a two-step process and exclude oligomers and protofibrils as obligate intermediates in amyloid fibril assembly (48, 49). However, in the absence of high-resolution images for both intermediate species and mature fibrils, the connection between the mechanism of amyloid formation and protein folding remains tenuous.

![Nucleated Conformational Conversion (NCC)](image)

**Figure 2.4:** A schematic representation of the nucleated conformational conversion model. Smooth circles represent aggregated-state proteins; jagged circles represent soluble-state proteins; and open jagged circles denote possible conformational heterogeneity. Adapted from Serio *et al.* (47).

Furthermore, a recent report suggest that amyloid fibrils are polymorphic (50). That is, a single polypeptide chain can assume an array of fibril conformations. It is thus probable that by virtue of variations in experimental conditions, a polypeptide chain may be confronted by Levinthal’s paradox (51). In this case however, a range of conformational transitions leading to amyloid fibril assembly are explored. For example, Goldsbury *et al.* (48) reported the possibility of multiple assembly pathways in Aβ fibrillation.

In summary, it is generally accepted that protein aggregation is triggered by the destabilization of the native structure, resulting in a structural reorganisation that exposes buried hydrophobic segments of the polypeptide chain to the aqueous environment. The solvent-exposed hydrophobic groups coalesce into oligomeric aggregates, via hydrophobic interactions, as an inherent disposition to sequester the exposed hydrophobic
groups. The aggregates grow in size, and in doing so, undergo a conformational transition into fibril competent nuclei. Provided a sufficient number of fibril nuclei develop, polymerisation proceeds rapidly leading to the formation of the thermodynamically stable fibril structure.

2.5 Accessories to protein aggregation

Several factors, in vivo and ex vivo, have been found to mediate the rate of protein aggregation and also influence the stability of amyloid fibrils. For example, the deposition of misfolded proteins as amorphous aggregates and/or amyloid fibrils in vivo is believed to be influenced by extraneous proteinaceous elements such as apolipoprotein E, heparin sulphate proteoglycans and serum amyloid P-component (11), and more recently, non-proteinaceous elements such as biometals (52). These additional non-amyloid elements are found co-precipitated in senile plaques in several neurodegenerative diseases including Alzheimer’s disease. It is believed that knowledge of the contributions of these accessory factors to protein aggregation will inform the development of novel diagnostics and therapeutic strategies to identify and counter the debilitating effects of protein conformational disorders.

In vitro studies have shown that physical parameters such as temperature, pH and agitation influence the kinetics of protein aggregation (53, 54). Interestingly, while agitation is a common feature of experimental protocols employed in examining protein aggregation, its contribution to the aggregation process is ill understood.

Mechanical perturbation of protein solutions via stirring, shaking, sonication, vortexing and pumping, initiate fluid flow which in turn generates hydrodynamic shear stress in the protein solutions. It is believed that the fluid drag associated with shear flow may destabilize the native structure of protein molecules, leading to denaturation and subsequent aggregation (9). Given that protein solutions have the inherent disposition to flow, and that shear flow is a common feature in protein denaturation/aggregation protocols, it is essential to understand the influence of fluid forces on the structural integrity of aqueous protein systems.
2.6 Effects of hydrodynamic drag on protein structure and function

The effect of fluid shear stress on protein structure is a subject of practical interest because it is a common phenomenon in bioprocessing (55) and microcirculation (56). A variety of biopolymers, including proteins, have been shown to exhibit conformational dynamics in shear flow (57-59). Protein solutions are exposed to shear stresses during bioprocessing steps as a result of centrifugation, fractionation, pumping, and ultrafiltration (55, 60, 61). Similarly, the shipping and handling of biotherapeutics and protein based bioprocessing reagents such as monoclonal antibodies, hormones, cytokines and enzymes can result in significant agitation. For these reasons, an understanding of the effects of shear flow on protein stability, and a means of testing and measuring this effect, would allow the appropriate design and selection of manufacturing processes, conditions and formulations which would ensure maximum yield and stability.

In physiology, protein components of blood plasma, experience a combination of confinement, due to the crowding of biological molecules (e.g. lipids, carbohydrates) in the cellular environment, and shear rates of up-to $10^5$ s$^{-1}$ during microcirculation (62, 63). Interestingly, alterations in haemodynamic shear stress is thought to trigger the onset of protein conformational disorders such as Alzheimer’s disease (64, 65), and the pathogenesis of vascular disorders including atherosclerosis (13, 66). It is noteworthy that both disease states involve the deposition of misfolded proteinaceous material as plaque (13, 64, 65). It is anticipated that understanding the rheology of protein systems might help reveal the possible contribution of haemodynamic drag to protein conformational disorders.

While there exists a significant body of literature on the rheological properties of protein solutions, these reports give conflicting results which implies that a detailed understanding of the conformational dynamics of protein molecules in a shear-driven flow field remains elusive (67, 68).
2.6.1 Studying the effect of shear flow on proteins

Extensional flow vs. simple shear flow
The common flow fields applied in shear studies are extensional flow and simple shear flow. A homogeneous extensional flow, also called elongational flow or stretching flow, is characterized by a linear velocity gradient of the form \( v_y = \gamma y \) along the direction of flow (69). The strain rate, \( \gamma = \frac{\partial v_y}{\partial y} \), is constant. An example is the flow of a protein solution through a converging channel with hyperbolically-shaped walls. On the other hand, simple shear flow describes fluid flow characterised by a velocity gradient perpendicular to the flow field. It is modelled as a linear superposition of rotational flow with vorticity \( \omega \), and elongational flow with strain rate \( \gamma \) (58). In the rotational component of the flow field, protein molecules experience whole body rotation with no hydrodynamic shear strain, hence maintain their structural integrity. However, protein molecules are subjected to stretching events when oriented in the extensional flow field. The hydrodynamic shear stress resulting from these events is thought to destabilize the native protein structure, leading to aggregation (9). The mathematical relationship between shear stress (\( \tau \)) and velocity gradient (\( \gamma \)) in a given flow field is expressed as \( \tau = \eta \gamma \) (70). A tangential stress of this type is called shearing, where infinitesimally thin layers of fluid slide over each other in laminar flow. Shear stress has units of force per unit area (N/m²), and is a measure of the actual hydrodynamic drag acting on the protein solution under flow. The velocity gradient, also referred to as shear rate or strain rate, has units of inverse time (s⁻¹).

It is noteworthy that the magnitudes of rotational and elongational components of simple shear flow are equal (\( |\omega| = |\gamma| \)). This implies that the conformational dynamics of protein molecules in the flow field are dictated by the random occurrence of both events. In practice, the rotational and elongational components of flow differ in magnitude.
2.6.2 Devices for generating shear flow

In many protein studies, shear flow is generated by subjecting protein solutions to flow patterns characterized by uniform or heterogeneous velocity gradients (60). Shear devices that create a near uniform velocity gradient through protein solutions provide a well controlled and quantifiable shear stress. Hence, such devices are ideal for studying the effects of shear forces on protein molecules. Conversely, heterogeneous velocity gradients, e.g. resulting from stirring or shaking, provide poorly controlled shear conditions that are difficult to quantify. It is worth noting that the differences in experimental devices applied in shear studies, and the associated data interpretation, could be a major contributory factor to the inconsistent experimental results reported throughout the literature.

Several studies that show the effect of shear flow on a number of protein systems, along with the shear devices employed, are summarized in Table 2.1. The experimental flow devices used for protein studies fall into two main categories; capillary and rotational flow devices.
Table 2.1: Studies of the shear/stability of protein systems using a variety of shear devices.

<table>
<thead>
<tr>
<th>Device geometry</th>
<th>Protein</th>
<th>Result</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Couette</td>
<td>Insulin</td>
<td>Unfolding and aggregation</td>
<td>(71)</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td></td>
<td>Unfolding</td>
<td>(72)</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td></td>
<td>Fibril formation</td>
<td>(54, 73)</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td></td>
<td>Unfolding</td>
<td>(74)</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td></td>
<td>Unfolding</td>
<td>(75)</td>
</tr>
<tr>
<td>R-phycoerythin</td>
<td></td>
<td>Unfolding</td>
<td>(76)</td>
</tr>
<tr>
<td>Amyloid-β</td>
<td></td>
<td>Fibril formation</td>
<td>(77)</td>
</tr>
<tr>
<td>Taylor-Couette</td>
<td>Lysozyme</td>
<td>Unfolding</td>
<td>(78)</td>
</tr>
<tr>
<td>Cytochrome-c</td>
<td></td>
<td>Unfolding</td>
<td>(78)</td>
</tr>
<tr>
<td>Cone and plate</td>
<td>α-amylase</td>
<td>Loss of activity</td>
<td>(79)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td></td>
<td>Degradation</td>
<td>(80)</td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
<td>Loss of activity</td>
<td>(81)</td>
</tr>
<tr>
<td>Glycoprotein Ib &amp; IIb-</td>
<td></td>
<td>Unfolding</td>
<td>(82)</td>
</tr>
<tr>
<td>IIIa</td>
<td>von Willebrand factor</td>
<td>Aggregation</td>
<td>(83)</td>
</tr>
<tr>
<td>Concentric cylinder</td>
<td>Urease</td>
<td>Particle formation</td>
<td>(84)</td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
<td>Particle formation</td>
<td>(84)</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td></td>
<td>Loss of activity</td>
<td>(85)</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td></td>
<td>No change</td>
<td>(86)</td>
</tr>
<tr>
<td>Growth hormone</td>
<td></td>
<td>Unfolding and fragmentation</td>
<td>(86)</td>
</tr>
<tr>
<td>Coaxial cylinder</td>
<td>Urease</td>
<td>Loss of activity</td>
<td>(7)</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td></td>
<td>Particle formation</td>
<td>(85)</td>
</tr>
<tr>
<td>Capillary</td>
<td>Remnet</td>
<td>Loss of activity</td>
<td>(6)</td>
</tr>
<tr>
<td>Urease</td>
<td></td>
<td>Particle formation</td>
<td>(84)</td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
<td>Loss of activity</td>
<td>(6)</td>
</tr>
<tr>
<td>Cytochrome-c</td>
<td></td>
<td>No change</td>
<td>(67)</td>
</tr>
<tr>
<td>Microfluidic cell</td>
<td>von Willebrand factor</td>
<td>Unfolding</td>
<td>(68)</td>
</tr>
</tbody>
</table>

**Capillary/microfluidic flow devices**

Capillary flow occurs when a fluid is forced through a conduit of known dimensions by applying a given pressure difference between the inlet and outlet of the conduit (87). Here, the fluid velocity profile is of the Poiseuille type (non-homogeneous shear flow) with the maximum shear rate at the fluid-vessel boundary, reducing gradually towards the vessel centre. Very high shear rates, up to $10^5$ s$^{-1}$, can be achieved but over short residence times on the order of milliseconds (27, 88). It is noteworthy that the short residence time spent in the flow field could contribute to incomplete protein stretching.
However, recirculation of protein solutions through conduits have been successfully applied to increase residence times (80), offering a good model system for fluid shear stress in ultrafiltration units and membranes.

**Rotational flow devices**

Cone-and-plate and concentric-cylinder viscometers are two types of rotational flow devices commonly used to impart shear stress on protein systems across a narrow gap. Both models consist of two parts where one rotates with the other being stationary. In the case of the concentric-cylinder, also known as Couette or coaxial cylinder flow-cell, there are several sorts that differ in basic design but operate on the same principles (90). However, a through-cell Couette arrangement is ideal for generating a simple shear flow, as the design minimizes any end effects (91). This type of device also curtails particle settling or migration away from the solid boundaries, thereby limiting variations of fluid properties across the gap (92). In addition, a nominal shear rate can be calculated for each rotational speed. At high rotational speeds, large deviations from the ideal flow may occur due to the formation of vortices. It is recognized that vortices occur in the extensional component of shear flow and should therefore be avoided if a simple shear flow is desired (93). The advantage of rotational shear devices over that of the capillary design is that the entire test solution is sheared at a near constant rate. This permits the study of any time-dependent behaviour of protein samples over an extended period of time. In the through-cell Couette arrangement, the shear rate can be regarded as constant only if the gap width is much less than the radius of the inner cylinder (94). In the cone-and-plate design, the solution is contained in the space between a cone of large apex angle and a plate with a flat surface normal to the axis. For small angles between the cone and plate, the shear rate is considered constant for all radial positions.

### 2.6.3 Protein function

Preliminary studies on the relationship between shear flow and protein structure exploited the catalytic activity of enzyme solutions as a means of measuring protein integrity. Thus, a decrease in enzyme activity implied a structural deformation of the native enzyme molecules. This method of investigation presented a far less complicated approach
relative to studying the structural dynamics of non-enzyme proteins in shear flow. Indeed, these early studies were critical in defining the appropriate, and operation of, devices to allow the study of the behaviour of proteins in shear flow; allowing the estimation of shear forces required to perturb protein function with insight into possible resulting structural changes.

Studies on catalase, rennet, and carboxypeptidase solutions sheared in a narrow gap coaxial viscometer (6) at shear rates between 9.15 and 1155 s$^{-1}$ for 90 minutes, showed a strong correlation between decrease in enzyme activity and shear (shear rate $\times$ time). A loss of enzyme activity occurred for shear greater than $10^4$ although the rennet solutions partially regained activity upon the cessation of shearing. The observed enzyme inactivation was attributed to the breaking of the molecule’s tertiary structure when oriented appropriately in the shear field. The authors discounted any significant contribution from the air-liquid interface in the viscometer, where protein molecules, by virtue of their amphipathic nature, may aggregate (causing inactivity). It is also possible that the loss in enzyme activity was due to a flow-induced limited residence time for enzyme-substrate interactions. Further studies on plasma fibrinogen revealed a loss in its clot formation capability when exposed to shear flow in model ultrafiltration systems (80).

Studies on the shear-stability of urease in a coaxial cylinder viscometer, allowing direct monitoring of urease catalysed hydrolysis of urea during shear exposure, showed continuous decrease in the rate of urease activity with increasing shear rate (48, 288, 741, and 1717 s$^{-1}$) (7). The partial deactivation of urease revealed both reversible and irreversible elements. The reversible inactivation was ascribed to hydrodynamic distortion of the enzyme’s structure in flow, whereas permanent inactivation was attributed to the breaking of the enzyme’s tertiary structure. The permanent loss in urease activity correlated with shear strain, with a critical shear value of $10^5$, similar to that previously reported (6). Studies on catalase degradation in streamline and turbulent flow regimes showed a significant inactivation in the latter where a vortex and air bubbles had formed in the solution (95). This suggested that the resulting air-liquid interface may
have contributed significantly to catalase degradation. It was also shown that shear stress in a microfiltration membrane system caused a significant decrease in the catalytic activity of yeast alcohol dehydrogenase (ADH) (96). This observation was attributed to membrane-enzyme interactions resulting from a shear-induced deformation of the enzyme structure.

In studies of the shear-stability of alcohol dehydrogenase solutions using a high shear concentric viscometer, a rotating disk reactor and several pumps, it was found that fully active tetrameric molecules of ADH showed negligible change in activity even for strain rates as high as 26,000 s\(^{-1}\) over 1 h in the high shear device (97). Turbulent regimes in the rotating-disk reactor (3600 rpm for 5 h) and up to about 1500 passes in the various pumps likewise had little effect on ADH activity. However, the introduction of a gas-liquid interface in the rotating-disk reactor resulted in a 40% decrease in the initial ADH activity after 5 h. Similarly, a significant decrease in ADH activity was observed in a gear and a Jabsco pump after 2000 passes. This report suggested that interfacial denaturation, and not shear \textit{per se}, accounts for the deformation of proteins in solutions exposed to shear flow, and that the native conformation of globular proteins, when in a chemically stable environment, are stable in shear flows usually encountered in practice.

The importance of an air-liquid interface in promoting shear-induced protein denaturation was shown in a study with progesterone 11\(\alpha\)-hydroxylase complex, using an open concentric cylinder viscometer and a horizontal viscometer closed at both ends (98). It was observed that the gas-liquid interface, in concert with high shear regimes which replenished the protein at the interface, promoted rapid protein denaturation as previously reported (97). In addition, a wall surface effect on the enzyme complex was observed as the gap width of the viscometer was reduced from 0.475 to 0.15 mm. Furthermore, it was observed that viscous enzyme solutions showed a marked decrease in activity as a function of shear stress (98). These observations lead the authors to propose that the applied shear stress resulted in enzyme denaturation via either the rupture of the enzyme membrane or the enhanced solubilisation of component(s) of the enzyme complex by the velocity gradient in the agitated solution.
The above studies clearly showed that shear could have a profound effect on the activity of enzymes, and by inference on protein structure. The shear-effect was more pronounced in the presence of an air-liquid interface. A key limitation of the above studies was that any deduction of structural change was based on indirect evidence derived from enzyme activity. Indeed, whereas shear may not have caused significant structural damage, a decrease in enzyme activity could result from minor modifications to an enzyme’s active site. In many of the experiments, the enzymes were subjected to intermittent shearing force to allow sampling. Therefore, significant time would have elapsed between sampling of solutions and testing for enzyme activity. This would have allowed reversible structural changes to revert and thus go undetected. The advent of new experimental techniques, allowing real-time measurements of the solution conformation of proteins, is providing new insight into the shear-stability of proteins with respect to variations in the sensitivity of different proteins to shear, and the specific molecular changes occurring in response to shear exposure (59, 99).

### 2.6.4 Protein structure

Human von Willebrand Factor (vWF), a blood plasma protein with important functions in coagulation, was amongst the first of protein systems, besides enzymes, to be studied for shear induced structural changes. By using a combination of atomic force microscopy and a rotating disk system, vWF was shown to undergo a shear-induced conformational transition from a globular state to an extended chain conformation at a critical shear stress value $>31.5 \text{ dyn/cm}^2 \ (\sim 828.9 \text{ s}^{-1})$. The result was the exposure of intra-molecular domains of vWF. It was suggested that shear-induced structural changes in vWF may have an important role in platelet adhesion and thrombus formation in regions of high shear stress, as would develop at the site of a bleeding injury (100). To this point, it had been considered that clot formation was initiated through direct shear activation of platelets (62).

Recently, fluorescence microscopy has been used to probe vWF’s response to hydrodynamic stress in a microfluidic flow chamber (28). vWF was fluorescently labelled with Alexa fluor 488, which was attached to the primary amines of the
glycoprotein using tetrafluorophenyl ester. It was found that shear rates greater than 1000 s$^{-1}$ triggered the stretching of vWF in solution. In addition, real-time measurements of the conformational dynamics of vWF in response to laminar shear flow, in a Couette flow-cell, using small angle neutron scattering (SANS) spectroscopy, demonstrated that hydrodynamic shear stress triggered an irreversible conformational change in vWF within 10 minutes (75). The observed conformational change was more pronounced with increasing shear rate.

While studies of vWF provided clear evidence that shear flow can alter protein structure, it may be argued that different proteins experience different shear since the strain rates required to trigger structural alterations decreases with increasing molecular weight and solvent viscosity (27). For example, studies on the effect of high shear rates on ferric equine cytochrome c, a small globular protein of molecular weight 12,384 Da, showed no significant structural alterations when exposed to shear rates as high as 10$^5$ s$^{-1}$ in a capillary flow device (27). It was suggested that a shear rate of ~10$^7$ s$^{-1}$ would be required to denature a small globular protein in water. This contradicts the observed abrupt stretching of vWF (>20,000 kDa) in a microfluidic flow chamber at a threshold shear rate of 10$^3$ s$^{-1}$ (28). Clearly, the larger, more complex vWF would be expected to be more susceptible to shear stress relative to cytochrome c. However, it is noteworthy that specific conformational characteristics such as α-helix and β-sheet composition, and intra-molecular interactions (e.g. hydrophobic, electrostatic) and covalent bonds (e.g. disulfide), would be an important determinant of protein stability.

Thus, protein systems with comparable molecular weights may differ in their shear-stability due to differences in their solution properties. For example, previous studies on the effects of both high shear (>10$^7$) and high shear rate (>10$^5$ s$^{-1}$) on recombinant human growth hormone (rhGH) (~22 kDa) and recombinant human deoxyribonuclease (rhDNase) (~31 kDa) showed that neither of the two solutions exhibited significant changes in their near and far-UV circular dichroism spectral profiles (86). However, rhGH showed changes in its melting temperature and the presence of low molecular weight fragments after prolonged shearing. Further studies showed that rhGH denatured
upon exposure to high shear in the presence of an air-liquid interface whereas rhDNase remained relatively stable (101). The stability of rhDNase was attributed to its comparatively high surface tension and low foaming tendency in solution.

Intriguingly, it has been shown that human and bovine albumin samples, which differ in only two amino acids, show different aggregation kinetics upon shaking at high shear rates (>8000 rpm) (102). In addition to the monomer-dimer transition observed in both albumin samples upon shearing, human serum albumin also showed trimer formation especially at high shear rates. Since the protein concentration and solvent-air interfaces were considered as fixed factors, the observed differences in the degree of albumin aggregation was attributed to the minor variation in their primary structure.

2.6.5 Protein aggregation
As has been discussed above, shear can result in protein denaturation and compromised function. It is also well established that denaturation of proteins can lead to aggregation (103). Hence, the use of agitation (uncontrolled shear stress) to both induce and accelerate protein aggregation and amyloid fibril formation is common practice throughout the literature (103). For example, under favourable solution conditions, various forms of agitation including shaking, sonication and stirring have been successfully applied to accelerate the aggregation of insulin (15, 30, 104-106), amyloid-β (77, 107), β2-microglobulin (108, 109), β-lactoglobulin (54), albumin (102) and the PDZ domain from murine Protein Tyrosine Phosphatase Bas-like (PDZ2) (110) among others. In one particular study, a range of structurally diverse protein systems including BSA, myoglobin, lysozyme, Tm0979, SOD and hisactophin were shown to generate amyloid like structures following sonication (111).

Shear effects on protein aggregation have also been studied in uniform flow fields. For example, a shear-induced nucleation, without flow enhanced aggregation, of preheated β-lactoglobulin solutions (0.5 wt%) at low pH has been reported (112). Thermal treatment of β-lactoglobulin solutions induced the formation of metastable pre-fibrillar aggregates, without which fibril formation was absent in the sheared samples. Shearing of β-
lactoglobulin samples was done either continuously or in short pulses; both producing a similar degree of fibril formation. These observations lead to the conclusion that brief mechanical perturbation of an aggregation prone protein solution was enough to enhance fibril formation. That is, shear flow only triggered the nucleation of fibril formation but did not influence the polymerization of pre-aggregates into mature fibrils; discounting orthokinetic coagulation under the experimental conditions. However, the fibrils formed from the continuous shear treatment showed a smaller variance in Gaussian length distribution relative to those from the pulse shear treatments, indicating a homogenizing effect of the continuous shear treatment. Evidently, a synergy of heat and agitation triggered rapid fibril formation. The experiments were performed in a cone and plate rheometer with Couette geometry at a moderate shear rate of 200 s\(^{-1}\) over 5 h (112).

Another study showed that the aggregation of preheated β-lactoglobulin solutions was shear rate dependent (54). In addition, if shearing was performed prior to thermal treatment, a rapid enhancement of fibrillar species still occurred, suggesting a shear-induced formation of the precursor nuclei required for fibril development. Interestingly, at shear rates >500 s\(^{-1}\), fibril degradation, possibly arising from the persistent stretching (extensional flow) events in the flow field, was observed. In later studies (113), it was reported that a sufficiently high β-lactoglobulin concentration (> 3 wt%) was required to initiate fibril formation when samples were heated and sheared simultaneously. In addition, the amount of fibrils formed increased as a function of shear rate up to 337 s\(^{-1}\) beyond which an opposing shear effect, similar to that reported earlier (54), was observed. The paradoxical shear effect was explained by a flow enhanced polymerization of the fibril nucleus in low shear regimes, whereas in high shear regimes, the extensional component of the flow field overwhelmed the nascent inter-β-strand hydrogen bonds stabilizing the aggregating fibrils. Furthermore, the authors observed that the shear applied produced shorter fibrils and also enhanced, below a critical fibril concentration, the viscosity of the resulting fibril solutions.

More recently, a similar flow enhanced fibril formation has been observed in aqueous solutions of bovine insulin (59), thermally treated amyloid-β (77) and β-lactoglobulin
samples. The shear-stability of bovine insulin in Couette flow was probed via a novel direct circular dichroism (structural) measurement, \textit{in situ} and in real time. Helical segments of native insulin were observed to unfold as a function of shear rate with fibrillar forms evolving in relatively strong shear regimes ($600 \text{ s}^{-1}$). The shear-effect on preheated amyloid-$\beta$ samples showed similar trends to that reported earlier (54). In the $\beta$-lactoglobulin studies, the authors compared the morphology and mechanical properties of fibrils formed under heterogeneous (stirred) and uniform (Couette) flow conditions. They found that $\beta$-lactoglobulin fibrils resulting from heterogeneous flow had twisted ribbon-like morphologies and higher mechanical strength relative to the beaded fibrils formed in Couette flow. Evidently, the intertwining of fibrils produced in the variable shear regime imparted additional mechanical stability to the overall fibril structure. It was hypothesised that polymerization of fibril seeds occurs in the flow field, and in the case of stirred samples, mature fibrils anneal into twisted ribbons in regions of less hydrodynamic drag.

Taking together, even if in some cases the initial structural destabilization required for protein aggregation is not triggered by shear, its ability to accelerate the polymerization of metastable prefibrillar aggregates into mature fibrils still has grave implications in bioprocessing and physiology.

Regarding the mechanism of shear-induced protein aggregation, there is a general consensus that mechanical perturbation of a protein molecule often results in a structural destabilization of the native conformation; leading to the exposure of sequestered hydrophobic residues to the surrounding medium. Solvent-exposed hydrophobic groups nucleate via hydrophobic interactions and subsequently aggregate. Several studies show that the initial destabilization of the protein structure occurs at the solvent-air or solvent-solid interface, where surface tension forces unfold the native protein (84, 85, 101, 115). The air-water interfacial force is estimated to be 140 pN (116), taking the air-water interface to be 2 nm in depth with a 0.07 N/m surface tension (117). This value is comparable to the 150 pN observed to unfold globular proteins in atomic force microscopy studies (118). In addition, as agitation increases the turnover of the air-liquid interface, and thus the number of protein molecules interacting with this interface, the
nucleation of solvent-exposed hydrophobic species via hydrophobic interactions, and hence the aggregation of the bulk protein solution, increases dramatically (107). In fact, the influence of agitation can be so profound that, in the case of insulin, it was found to attenuate the effects of other parameters, such as protein concentration and ionic strength, that influence the kinetics of amyloid fibril assembly (15).

2.6.6 Molecular models and theoretical aspects
The complexity of protein molecules with respect to conformational heterogeneity as well as intra and intermolecular interactions between amino acid residues, with the consequent differences in the flexibility of secondary conformations and tertiary configurations between proteins, results in variations in their solution properties in shear flow. Indeed, differences in the levels of association in diverse protein systems dictate the different degrees of shielding, especially of hydrophobic residues concealed in the protein matrix, from hydrodynamic drag during shear flow (24, 119). Hence, it is difficult to obtain uniform data to allow the formulation of a theoretical model relating protein conformation to shear. For these reasons, dilute solutions of homopolymers, especially unbranched polymer chains, have been used as model systems for shear studies because of their inherent structural uniformity.

By virtue of its conformational plasticity in response to temperature, pH, salt concentration and alcohol content in solution (72), poly-L-lysine is commonly used as a model system in protein studies (120). The homopolyptide exists as a random coil at neutral pH, α-helix at high alkaline pH and β-sheet at temperatures >30°C (121). For these reasons, poly-L-lysine is the ideal choice in probing the initial conformational transitions, under denaturing conditions, between the three basic protein secondary conformations, namely; random coil, α-helix and β-sheet.

Experimental studies on the shear-stability of poly-L-lysine solutions (437 kDa), in an initial α-helical conformation, showed an increase in solution viscosity and turbidity with shearing time (122). A combination of Raman spectroscopy (collected in situ) and circular dichroism were employed for data acquisition in a Couette flow cell at 150 s⁻¹. In
addition, a flow induced-gelation and an increase in the β-sheet content of the samples was observed in solution concentrations >0.3 g/dL. However, the circular dichroism data further revealed a reversal of the α-helix to β-sheet conformational transition after prolonged shearing (>50 minutes). The observed conformational change and subsequent aggregation of the sheared samples was thought to involve flow-enhanced hydrophobic interactions between individual poly-L-lysine molecules in solution. Further studies, via real-time circular birefringence measurements, showed a reversible, shear-induced, helix-to-coil transition of poly-L-lysine in simple shear flow (72). The conformational transition was observed at a critical strain rate of 300 s\(^{-1}\), and the change was attributed to a shear-induced breakage of intramolecular hydrogen bonds in α-helical poly-L-lysine.

More recently, the molecular-weight-dependence of the shear-induced unfolding of α-helical poly-L-lysine has been measured in real-time using circular dichroism spectroscopy (see chapter 7) (99). It was found that the shear-stability of the helices increased as a function of molecular weight. The hysteresis observed in the long chains was attributed to the large network of hydrogen bonds (cohesive force) stabilizing the helix structure, and the associated hydrodynamic screening of helical segments from the drag in the flow field. Interestingly, irrespective of the molecular weight of poly-L-lysine, unfolding of the helical segments was found to occur at a critical strain (\(\dot{\gamma} \tau_c\)) value of \(\approx 10^5\), similar to that observed in globular proteins (6, 7). For this reason, it was proposed that the shear rate is not as critical as the duration of its application, which makes the idea of a critical shear rate arbitrary. In addition, the helix content \(\alpha\), was found to show a power law dependence with strain: \(\alpha \sim (\dot{\gamma} \tau)^{-1/2}\). Jaspe and Hagen (27) suggests that coil-stretch transitions readily occur in homopolymer systems because the free energy cost of unfolding is negligible compared to that of a native protein, and this was purported to explain why an extraordinarily high shear rate (10\(^7\) s\(^{-1}\)) is required to destabilize the latter. Others (25) have estimated a destabilizing strain rate in the region of 10\(^8\) s\(^{-1}\) for collapsed polymers in planar shear flow.
Classical theories on the shear-stability of polymer chains predict that a polymer chain would remain in a coil state in shear flow until a critical velocity gradient, which triggers an abrupt unwinding of the coil, is reached (24). In addition, abrupt conformational transitions are not expected in simple shear flow since the occurrence of extensional strain, hence polymer stretching, is random. It is hypothesized that at very high strain rates, where polymer chains spend a relative short time (residence time) in an extensional flow field, no unfolding or incomplete chain extensions may occur (89). These predictions were substantiated in later studies that simulated polymer dynamics in shear flow. For example, in molecular dynamics simulations of a grafted collapsed macromolecule in strong uniform flow, it was observed that a flexible macromolecule remains compact below a critical shear value but shows a globule-stretch transition above this critical shear value (123). The transition was reported to be more pronounced in strong extensional flow.

Lemak et al. (124) later compared the conformational instabilities of a minimalist β-barrel protein in both uniform and elongational flow fields using molecular dynamics simulations. It was reported that whereas unfolding was abrupt in elongational flow, the uniform flow fields presented a multi-step unfolding process, with several intermediates, depending on the tethered terminus of the protein. In explaining this observation, it was proposed that whereas every monomer (i.e. amino acid residues) experienced a destabilizing hydrodynamic drag in elongational flow, only the terminal monomer experiences this force in uniform flow. Hence, in uniform flow, deformation of the protein follows an unzipping mechanism where the β-strands are unfolded one at a time; leading to the evolution of intermediate states. More importantly, it was evident that the nature of the applied external force dictated the preferred mechanism for protein unfolding.

Szymczak and others observed similar flow effects on simulating the flow-induced stretching of integrin and ubiquitin (125, 126). They noted that the hydrodynamic drag on a grafted protein chain in extensional flow increases along the chain length as the polymer unfolds. Therefore, the initiation of unfolding triggers a positive feedback.
mechanism which leads to the rapid unravelling of the entire chain to the exclusion of intermediate states. Indeed, it had previously been predicted that the fluid drag acting on an initially coiled polymer increases as the molecule unfolds due to the decreased hydrodynamic interactions between its constituent monomers (24, 127). In addition, the authors observed that whereas the grafted protein chains unfold through a series of intermediate states in uniform flow, consistent with that reported earlier (124), synthetic helices unravelled smoothly with no detectable intermediate states (125). This highlights the fact that proteins and homopolymers indeed differ in their hydrodynamic properties in shear flow. Furthermore, it was also noted that the array of metastable conformations observed during protein unfolding in uniform flow was absent in mechanical pulling measurements in a force clamp apparatus such as the atomic force microscope.

A subsequent study examined the influence of hydrodynamic interactions, which facilitate the folding of protein molecules into compact native structures, on the mechanical stretching of proteins using ubiquitin as a model system (126). Mechanical stretching at constant speed, at constant force, and through fluid flow was examined. It was found that hydrodynamic interactions (cohesive forces) between amino acid residues facilitated unfolding (stretching) when a constant speed or force was applied to grafted ubiquitin. This observation was explained on the basis that an amino acid residue, which is pulled away from the bulk protein, creates a flow which drags other residues with it. In contrast, these cohesive forces were found to counter fluid forces by screening monomers concealed in the protein matrix from the drag in the flow field, thereby hindering ubiquitin unfolding in both uniform and elongational flow fields (128). It has been reported that hydrodynamic interactions increase with increasing protein size and hence influence the critical shear rate required to unfold a protein of radius $R$ (25). However, the authors note that this prediction pertains to solutions with high solvent viscosity and low interfacial tension.

Recently, Sulkowska and Cieplak applied the coarse-grained molecular dynamics model to investigate the resistance of 7,510 proteins to mechanical stretching at a constant speed (129). Pulling was done by the termini to determine the maximum force of resistance,
$F_{\text{max}}$, of the protein chains. They restricted their study to non-fragmented protein chains deposited in the Protein Data Bank by August, 2005 and between 40 to 150 amino acid residues long. For specific sequential lengths of proteins, the average $F_{\text{max}}$ increased with increasing chain length. That is, longer protein chains showed better resistance relative to shorter chains. In relation to conformational classes, $\alpha$-proteins were found to be weak and generated small $F_{\text{max}}$ values relative to $\beta$- and $\alpha\beta$-proteins. Specifically, immunoglobulins and transport protein homologies were shown to yield larger $F_{\text{max}}$ values. However, the authors acknowledged that proteins with multi-$\alpha$-domains may show a significantly high resistance to mechanical stretching. In total, 134 proteins were considered to be strong with a threshold force of 170 pN. The authors proposed that the mechanical strength of the strongest proteins arises from a clamp consisting of long parallel $\beta$-strands which are stabilized by a network of intra and intermolecular interactions.

### 2.6.7 Implications in physiology and bioprocessing

Proteins show a strong conformation-function relationship with destabilization of the native protein structure leading to dysfunctional, aggregation-prone species with undesirable effects (8). Reports in the literature demonstrate that shear, which is a common stress in both physiology (56, 62, 130) and bioprocessing (55, 60), can induce protein deformation (14). Therefore, it is important to analyze the relevance of shear stress to protein molecules under physiological flow and in bioprocessing.

Pulsatile blood flow generates non-uniform shear stress throughout the arterial system during circulation (130). Arterial shear rates peak at $1640 \text{ s}^{-1}$ (130), reaching $10^4 \text{ s}^{-1}$ (62) in partly clogged coronary arterioles. The shear stress generated on arterial walls is associated with the pathogenesis of atherosclerosis through the modulation of endothelial function (131). A characteristic hallmark of this disease state is the deposition of amyloid-like protein aggregates as plaque on arterial walls. Arterial amyloid-plaques may trigger vascular inflammation, weaken the elasticity of blood vessels, and alter biochemical reactions such as lipid metabolism (13). Interestingly, it has been shown that molecular confinement enhances the deformation of entangled polymers under squeeze
flow (132); a condition which models pulsatile blood flow. It is noteworthy that protein conformational diseases and vascular disorders have received considerable attention in research. However, the underlying mechanism(s) leading to these debilitating diseases remain to be fully understood. Therefore, the possible contribution of physiological shear stress to the progression of these diseases needs to be given attention.

In the case of bioprocessing, shear stresses are present during the separation, purification, shipping and handling of protein based products (55). For example, processing steps involving ultrafiltration reactors (133, 134), stirred tanks (135, 136), homogenizers (137) and pumps (97, 138) induce high shear stresses. Simulated lobe pump studies have shown that high shear stress in these processing steps is sufficient to cause minor changes to protein structure (138). These structural changes may trigger the exposure of hitherto buried hydrophobic regions to the aqueous environment; initiating intra/intermolecular hydrophobic interactions leading to aggregation. The simulation further suggests that shear stresses may have played a crucial role in the protein aggregation behaviour observed in a previous lobe pump study (139). Indeed, protein aggregation and particle formation during processing can affect the efficiency of protein recovery, efficacy, safety, and product shelf-life (140). Furthermore, therapeutic protein aggregates are associated with a range of clinical side effects including non-specific anti-complementary activity leading to anaphylactic shock (141, 142). In addition, insoluble aggregates can cause immune responses, lodge in the lung capillary bed, and block blood vessels (143-145).

The design of experiments to establish the effect of fluid shear stress alone on protein structure and function has proved difficult. Existing shear devices generate other stresses including surface interactions and enhanced protein-air contacts, which complicates data interpretation (134, 146, 147). However, it has been established that the shear-stability of a protein molecule depends on; (i) its primary structure and molecular weight (ii) the magnitude of shear strain and the duration of its application, and (iii) the viscosity of its surrounding medium (101, 102, 148). Therefore, these factors must be taken in consideration for a complete understanding of the shear-stability of protein systems. Advancing knowledge in this field will be crucial for the design of processes, especially
in the bioprocessing industry, to ensure protein stability, maintain functionality and promote process yield.

### 2.6.8 Future challenges

Future development of protein based products will increasingly require several areas of improvement, especially in-process-monitoring of the stability of protein solutions. This will require the development of experimental techniques to allow the measurement of shear stress on proteins of interest. This is imperative as several studies show that individual protein systems demonstrate unique flow properties in a given flow field.

Additionally, it will be essential to appreciate the shear \( (\dot{\gamma}t) \) thresholds beyond which particular protein systems destabilize in solution. This will inform the development of new processing equipment to improve efficiency during production. It is noteworthy that knowledge from such studies could contribute to our understanding of the purported shear-induced pathogenesis of vascular and protein conformational disorders \textit{in vivo}.

### 2.6.9 Summary

Protein unfolding and aggregation is an issue in both \textit{in vitro} and \textit{in vivo} situation, specifically in bioprocessing and in the pathogenesis of disease. Shear rates of less than \( 10^3 \text{ s}^{-1} \) have been found to significantly alter the three-dimensional structure of globular proteins, leading to aggregation and amyloid fibril formation. The strain history \((\dot{\gamma}t)\) is found to be an important parameter in the shear-effect. Therefore, even in environments where ostensibly low shear is generated can lead to shear-induced protein structural change. Given the diversity of protein species, and the diversity of molecular sizes, conformations and nature of intramolecular interactions, the susceptibility of proteins to shear can vary. It is clear that shear generating processes should be avoided in bioprocessing. Shear also exists in the circulatory system and could be a significant contributor to the pathogenesis of certain diseases through contributing to the aggregation of aberrantly folded proteins.
2.7 Peptide and polypeptide systems used in this study

The sample systems used in this study were amyloid-β, bovine insulin and poly-L-lysine. These are well characterized systems that are widely used in studies of protein denaturation, aggregation and amyloid fibril formation (12, 15, 149).

2.7.1 Amyloid-β

Amyloid-β (Aβ) is a peptide composed of 39-41 amino acid residues, and is a major constituent of the precipitated proteinaceous elements found in senile plaques of the Alzheimer’s disease brain (29, 150). The generation of Aβ isoforms in vivo is a normal metabolic process that involves the cleavage of a much larger metalloprotein, amyloid precursor protein (APP), through the synergistic action of β- and γ-secretases (31). These peptides occur in the plasma and cerebrospinal fluid (CSF) at low nanomolar concentrations, and are purported to play a physiological role as antioxidants (151) and endogenous regulators of synaptic excitability (152).

The most common Aβ isoforms in the CSF are Aβ40 and Aβ42. Aβ40 is the major soluble species, accounting for 90-95% of the Aβ peptides in normal individuals (153). Aβ42 is the second most abundant, making up ≤5% (153). Interestingly, it is rather the minor Aβ42 that is heavily enriched in amyloid plaques and is believed to be the most neurotoxic of the isoforms (154). The length of the hydrophobic C-terminal domain of Aβ has an impact on its self-aggregating properties and neurotoxicity. For example, it has been reported that the additional two residues in Aβ42 increases its hydrophobicity relative to Aβ40, thereby enhancing its propensity to fibrillate (155). The widely held view that fibrils formed from synthetic variants of the Aβ peptide in vitro are biochemically and structurally identical to those observed in senile amyloid plaques allows for extrapolation of in vitro studies to the in vivo situation (156, 157).

2.7.2 Bovine insulin

Insulin is a 51-amino acid residue peptide-hormone involved in the regulation of blood glucose levels. It is composed of two peptide chains, A and B, of 21 and 30 amino acid residues respectively (158). The A-chain contains an intrachain cystine bridge between
residues A<sup>6</sup> and A<sup>11</sup> and is covalently linked to the B-chain via two interchain cystine bridges, A<sup>7</sup>–B<sup>7</sup> and A<sup>20</sup>–B<sup>19</sup> (159). The peptide-hormone derives its stability from these strongly hydrophobic cystine groups (160). The secondary structure of native insulin is reported to be 58% α-helical with a 6% β-sheet region (161). The helical segments span residues A<sup>1</sup>–A<sup>9</sup> and A<sup>12</sup>–A<sup>19</sup> of the A–chain, and B<sup>9</sup>–B<sup>19</sup> of the B–chain (162-164). Others suggest an additional, phenol promoted, helix spanning residues B<sup>1</sup>–B<sup>8</sup> (165). In solution, insulin has the unique trait of assuming different association states including dimers, tetramers and hexamers (166). The various association states are dependent on the overall net charge on the protein and hence dictated by the solution pH. Insulin is hexameric at physiological pH, dimeric in mineral acids including HCl and monomeric in 20% acetic acid (159, 166). Insulin is conformationally flexible (between R- and T-states) at physiological pH but stable under acidic conditions (167).

Since its discovery in 1921, insulin has been isolated and produced in commercial quantities to treat insulin-dependent diabetics (168). The problem confronting industry is insulin aggregation during processing and delivery of the protein-drug. Conventional processing of insulin involves exposure of the peptide-hormone to agitation, low pH, shear flow (during filtration, pumping and centrifugation), and an array of interfaces during administration (105, 169). These conditions are known to facilitate conformational destabilization leading to partial unfolding and subsequent coalescence of insulin into amorphous and/or fibrillar aggregates (15). Indeed amyloid plaques have been observed in insulin-dependent diabetics after repeated administration of insulin (170) and in normal aging (171).

2.7.3 Poly-L-lysine
Poly-L-lysine (PLL) is a homopolypeptide composed of L-lysine residues (172). Variable chain lengths, hence molecular weights, are commercially available.

Poly-L-lysine can assume the three basic solution conformations (random coil, α-helix and β-sheet) known to exist in proteins depending on solution conditions including temperature and pH (173). The pK<sub>a</sub> of the terminal ε-amino group in PLL is reported as
Hence, the lysyl chains are positively charged at neutral pH and uncharged if the solution pH exceeds the $pK_a$ value of 10. At neutral pH, PLL assumes a random coil conformation since the repulsive forces between the lysyl side chains leads to a highly solvated state. At high pH (>10), PLL is α-helical and readily converts to β-sheet, a hallmark of amyloid formation, at elevated temperatures (≥30 °C) (121, 173). It is noteworthy that even at elevated pH (11.6), a detectable increasing fraction of random coil is observed with increasing temperature in the range 4 to 20 °C (121).

By virtue of its conformational plasticity in solution, poly-L-lysine is commonly used as a model protein system for the analysis of structural changes in response to some perturbation (174). However, because PLL is a simple linear chain of lysine residues, it may not be the best model system for proteins. This is mainly due to the fact that proteins are heteropolypeptides with a complex network of intramolecular interactions, which leads to the co-existence of a variety of solution conformations per molecule (175).
3. Experimental Techniques, Apparatus and Applications

A number of complementary techniques were employed in this study to enhance the clarity of data interpretation. Specifically, a combination of spectroscopic techniques and atomic force microscopy were employed in these studies. These techniques are common in the experimental investigations of the conformational dynamics of protein systems in solution, as well as the morphology of protein aggregates. Details of the techniques and mode of application are given below.

3.1 Fluorescence spectroscopy

This is a versatile technique with practical applications in studies of the structure and dynamics of macromolecules that show luminescent characteristics (39). For example, it is applied in fluorescence anisotropy and confocal microscopy to acquire information on the dynamics and morphology of macromolecules (e.g. proteins) respectively. In particular, this technique has played a lead role in our understanding of protein folding, misfolding and aggregation (39, 176). For example, it has been successfully applied in (i) the structural analysis and characterization of globular proteins, (ii) measuring molecular distances via fluorescence resonance energy transfer (FRET), and (iii) tracking biochemical responses such as amyloid fibril formation (19, 177).

3.1.1 Principles

Fluorescence is a unique property of molecules (fluorophores) that emit light as a result of the absorption of electromagnetic radiation at a given wavelength (177). A schematic depiction of the photophysics involved in this process is commonly represented by the Jabłoński diagram in figure 3.1. The absorption of light triggers the excitation of component electrons of the fluorophore from the ground electronic state to a multiplicity of electronic energy levels in the excited state (i.e. \( S_0 \rightarrow S_1, S_2, \ldots, S_n \)). Here, the electrons populate one of several vibrational levels of an excited electronic state (39, 177). Fluorophores that attain very high excited electronic states typically dissipate the
excess energy by relaxation to the lowest vibrational level of $S_1$. This occurs via internal conversion and vibrational relaxation on a timescale between $10^{-14}$ and $10^{-11}$ s (177).

Figure 3.1: Simplified Jabłoński diagram of the photophysics of luminescence.

The electronic relaxation to the ground state then proceeds via two luminescent processes, fluorescence and phosphorescence. Fluorescence occurs when the excited molecule relaxes from the lowest vibrational level of the singlet excited state to the ground state ($S_1 \rightarrow S_0$), as this process is typically complemented by the emission of low energy photons. Since the energy of the absorbed light is typically higher than that of the emitted photons, the emission usually occurs at longer wavelengths relative to the wavelength of absorption. A typical fluorescence emission lifetime is $\sim10^{-8}$s (177).

The emission spectrum is independent of the wavelength of excitation, and its occurrence at longer wavelengths (lower energy) was first observed by Gabriel Stokes in 1852 (177). However, the wavelength of fluorescence emission is dependent on the energy gap between the ground state and the singlet excited state attained by the fluorophore. The fluorescence quantum yield, which is a measure of the efficiency of the fluorescence process, is influenced by the type of fluorophore and the conditions of its surrounding
medium (e.g. polarity) (178). The following equation summarizes the overall energy balance during fluorescence and also explains the typical occurrence of emission at longer wavelengths relative to the wavelength of excitation:

\[
E_{\text{flsc}} = E_{\text{abs}} - E_{\text{vib}} - E_{\text{solv.relax}}
\]  
(3.1)

- \(E_{\text{flsc}}\) = energy of the emitted photons
- \(E_{\text{abs}}\) = energy of the excitation light
- \(E_{\text{vib}}\) = energy dissipated during vibrational relaxation
- \(E_{\text{solv.relax.}}\) = energy spent in adjusting the orientation of the surrounding solvent

Alternatively, electrons in the singlet excited state can cross over to a triplet excited state (intersystem crossing) if the two energy states overlap. Relaxation from the lowest vibrational level of the triplet excited state (\(T_1\)) to the singlet ground state (\(S_0\)), when accompanied by the emission of light, is termed phosphorescence. Here, the emission occurs at longer wavelengths relative to that of fluorescence, and on a relatively slow timescale (10^3 to 10^0 s) (177).

It is noteworthy that non-radiative relaxation to the ground singlet state may occur as a result of external (e.g. collision deactivation) or internal conversion (177). In addition, the relatively short lifetime of fluorescence emission, to an extent, limits the occurrence of collisional deactivation, intersystem crossing and phosphorescence.

In cases where two or more fluorophores exists in the same sample, non-radiative relaxation may occur as a result of the transfer of the excited-state energy from one fluorophore (donor) to another (acceptor) (39, 177). This phenomenon is termed fluorescence resonance energy transfer. The occurrence of FRET is generally dependent on the following conditions: (i) the fluorescence emission spectrum of the donor fluorophore should overlap the absorption spectrum of the acceptor fluorophore; (ii) the two molecules should be in proximity (typically 10 to 100 Å); and (iii) the transition dipole orientations of the donor and acceptor fluorophores must be approximately
parallel. This phenomenon has been exploited as a versatile fluorescence technique with wide applications in the biological sciences. For example, FRET is employed in studies of the complex intermolecular interactions of multicomponent structures such as proteins, carbohydrates, nucleic acids, and lipids. In addition, it is also applied as a molecular ruler to probe the spatial distribution and assembly of polymers (e.g. protein complexes).

3.1.2 Application
A Varian Cary Eclipse fluorescence spectrophotometer (Melbourne, Australia) was used in this study. The fluorometer is equipped with a peltier thermo unit for stirring and temperature control. The light source is a high voltage xenon flash lamp operating at a frequency of 80 Hz. That is, it can collect 80 readings per second. The amount of light a sample is exposed to, and the duration of exposure can be set using the excitation/emission slit widths and average time controls in the instruments’ software. The slit widths used in this study were 10 nm, for both excitation and emission unless otherwise stated, and an exposure time of 1 s. The detector is a photomultiplier tube (PMT), positioned at right-angles to the path of light. The intensity of the detected light can be increased or decreased by adjusting the PMT voltage. A constant voltage setting of 600 V was used in this study.

The instrument was set to scan or kinetics mode for data collection. The scan mode allows the wavelength of excitation/emission to be set at a fixed value whiles collecting a scan of the emission/excitation spectrum over a range of wavelengths (e.g. 300 – 600 nm). The kinetics mode permits the setting of up to six excitation and emission parameters at fixed wavelengths. The advantage here is that multiple factors, such as intrinsic and extrinsic fluorescence profiles, can be probed simultaneously in a given sample. Under either mode, data can be collected at a set time internal over an extended period of time.

The analytical cell used for the fluorescence studies was a 1 cm quartz cuvette (Starna, Australia).
3.2 UV-Vis absorption spectroscopy
A significant number of compounds contain moieties (chromophores) that absorb light in the ultra violet (UV) or visible (Vis) region of the electromagnetic spectrum. The intensity of radiation absorbed varies as a function of frequency or wavelength with a characteristic peak position. The peak frequency/wavelength of absorption is unique to individual compounds as it is determined by the atomic and molecular composition of the compound (179). By virtue of its specificity, this phenomenon is exploited as an analytical technique to determine and quantify the presence of particular substances in solution. For example, proteins containing Trp residues show a characteristic absorption peak at ~280 nm. The amount of light absorbed at this wavelength, less the background spectrum, can be used in concert with other parameters to estimate the concentration of the protein system in solution.

3.2.1 Principles
Beer’s law states that the fraction of radiation $A$, absorbed by a solution is directly proportional to the concentration $c$ (M), of the absorbing species. Taking into consideration the distance the light travels through the sample $l$ (cm), as well as the molar extinction coefficient of the absorbing species $\varepsilon$ (M$^{-1}$ cm$^{-1}$), the law is usually written as:

$$A = \varepsilon lc$$

Therefore, by measuring the fraction of radiation absorbed as a function of frequency or wavelength, detection systems can provide data that can be used to estimate the concentration of the absorbing species, hence the bulk sample, in solution. It is important to point out that deviations from Beer’s law may occur as a result of both instrumental and intrinsic factors (177). These include deviations in molar extinction coefficients at high sample concentrations, light scattering by partially soluble chromophores that aggregate in solution, and the fluorescence or phosphorescence of the sample (177).
3.2.2 Application
A Varian Cary 3E UV-Vis absorption spectrometer (Melbourne, Australia) was used to quantify the concentration of protein and polypeptide samples used in this study. The instrument employs two light sources to generate the required radiation; one for visible and the other for ultra-violet light. The two lamps switchover at 350 nm. The spectrometer was set to scan in a single beam mode using a data interval of 1 nm, scan rate of 600 nm/min, 0.1 s response time, and a bandpass of 2 nm. Data was collected over the wavelength range of 200 to 350 nm using optically matched 1 or 2 mm pathlength quartz cells (Starna, Australia).

Background contributions to the absorbed radiation were taken into consideration by using the solvent spectrum of the prepared samples as a baseline for data collection.

3.3 Circular dichroism spectropolarimetry
Circular dichroism is a simple yet powerful quantitative structural technique commonly applied in studies of the solution conformation of proteins and nucleic acids (22). It has been particularly instrumental in advancing the structural characterization of an array of protein systems. Furthermore, it has been successfully applied in studies of the conformational transitions between secondary structural elements of proteins (e.g. α-helix ↔ β-sheet) in response to perturbation by temperature, pH, denaturants and shear flow (23, 59). More importantly, in the field of structural biology, circular dichroism has improved our understanding of the molecular interactions involved in protein folding and unfolding.

3.3.1 Principles
Circular dichroism measures the differential absorption of left- (L) and right-handed (R) circularly polarized light, by an optically active (chiral) chromophore in solution, as a function of wavelength (λ) (22). The chirality of the absorbing species may stem from its; (i) asymmetric structure (e.g. a tetrahedral carbon atom with four different substituents), (ii) covalent linkage to a chiral centre in the molecule, or (iii) presence in an asymmetric environment by virtue of the tertiary structure of the molecule (180). Chiral
chromophores absorb the two circularly polarized radiations to different extents, $|A(\lambda)_L| \neq |A(\lambda)_R|$, resulting in an elliptic wave. The two radiations sum-up to a single linearly polarized light which is sensed by linear detection systems in conventional CD instruments. In brief, generating a CD signal requires a chromophore which is in an asymmetric environment where $\Delta A(\lambda) \neq 0$. A schematic representation of this phenomenon is shown in figure 3.2.

Figure 3.2: Photophysics of circular dichroism. (A) Shows plane polarized light as a superposition of opposite circularly polarized light of equal amplitude and phase. (B) Depicts an elliptically polarized wave when the two radiations (L and R) are absorbed to different extents by a chiral chromophore.

Circular dichroism signals arise from electronic transitions resulting from the absorption of radiation in the near-ultraviolet, far-ultraviolet, and visible regions of the electromagnetic spectrum. Protein molecules absorb electromagnetic radiation from the near- and far-UV spectral regions, and this feature has been exploited in studies of their solution conformations.

In protein studies, absorption in the near-UV spectral region (typically 240 – 350 nm) is attributed to disulfide bonds and the aromatic side chains of amino acid residues (181).
Specifically, disulfide bonds absorb radiation throughout the near-UV region, giving broad weak signals between 240 to 300 nm (22), whereas the aromatic amino acid side groups absorb at ~245 nm (His), 250 – 270 nm (Phe), 270 – 290 nm (Tyr), and 280 – 300 nm (Trp) (22, 181).

Signals from these chromophores are highly dependent on the tertiary fold of the protein and thus sensitive to minute changes in the tertiary structure of protein molecules (180). That is, the tertiary fold of the protein molecule places these groups in an asymmetric environment, hence their optical activity. To emphasize this fact, aromatic side chains of unfolded proteins show an almost zero CD signal. Therefore, the detection of strong near-UV signals is indicative of a protein system with a well defined 3-D structure in solution.

It is noteworthy that the shape and intensity of signals in this region depend on the number of the absorbing species present as well as their surrounding microenvironment (e.g. polarity and rigidity) (180). For this reason, relatively high protein concentrations (≤ 2 mg/ml) are required for optimal sensitivity of the detector. In addition, the presence of disulfide bonds in a protein sample complicates data interpretation from the near-UV spectral region because of the overlapping of signals originating from disulfide bonds and that of the aromatic side chains (181). It is also important to point out that origins of signals in the near-UV region are not fully understood although this spectral region has been successfully applied as the fingerprint spot for the tertiary structure of proteins.

The far-UV spectral region of CD (180 – 240 nm) can provide quantitative information on the secondary structural elements of proteins (120). The main chromophore is the polypeptide backbone (peptide bonds) which show a weak but broad $n \rightarrow \pi^*$ electronic transition positioned in the region of 210 nm, and a more pronounced $\pi \rightarrow \pi^*$ electronic transition near 190 nm upon the absorption of radiation (23). For a polypeptide chain with regular secondary structure, the transition dipoles of neighbouring amide bonds interact, leading to an exchange of excitation energy between molecules. This interaction results in exciton splitting of the transition dipoles with consequent hypo- and/or hyperchromic shifts in their wavelength positions in the far-UV spectrum (182). Therefore, various solution conformations (e.g. unordered, α-helix and β-sheet) found in
proteins generate characteristic absorption bands with distinct shapes and magnitudes in the CD spectrum (figure 3.3) (22, 183).

![Figure 3.3](http://employees.csbsju.edu/~hjakubowski/classes/ch331/protstructure/olcompseqconform.html)

Recognizable bands from the solution conformation of proteins in the far-UV region, using poly-L-lysine as a model system, are summarized in table 3.1.

<table>
<thead>
<tr>
<th>Solution Conformation</th>
<th>Electronic transitions</th>
<th>λ(nm)</th>
<th>Δε (cm⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unordered</td>
<td>n → π*</td>
<td>217</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>π → π*</td>
<td>197</td>
<td>-12</td>
</tr>
<tr>
<td>α-helix</td>
<td>n → π*</td>
<td>222</td>
<td>-11</td>
</tr>
<tr>
<td></td>
<td>π → π*</td>
<td>208</td>
<td>-12</td>
</tr>
<tr>
<td></td>
<td>π → π*</td>
<td>190</td>
<td>+30</td>
</tr>
<tr>
<td>β-sheet</td>
<td>π → π*</td>
<td>218</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td>π → π*</td>
<td>195</td>
<td>+9</td>
</tr>
</tbody>
</table>
In summary, a predominantly random coil CD spectrum shows a small positive $n \to \pi^*$ transition at 217 nm and a large single negative $\pi \to \pi^*$ transition at 197 nm. A signal dominated by an $\alpha$-helical structure shows a large negative $n \to \pi^*$ transition at 222 nm, while the negative 208 nm and positive 190 nm bands result from the exciton splitting of the $\pi \to \pi^*$ transition. The CD spectrum of a $\beta$-sheet rich secondary structure also shows a split in the $\pi \to \pi^*$ transition with a negative band occurring at 218 nm and a positive band at 195 nm. It is worth noting that in certain cases, aromatic side chains as well as sulphur-containing residues, contribute significantly to this spectral region (181).

The far-UV CD spectrum serves as a fingerprint region for regular protein secondary structures. Hence, structural changes and conformational transitions that occur in protein molecules under stress (e.g. thermal treatment) can be followed and quantified by monitoring changes in this spectral region (180). A significant number of algorithms exist that can be employed to deconvolute CD spectra in order to analyze contributions from the component secondary structural elements of the sample of interest. Existing algorithms that are commonly employed include CDSSTR, CONTIN, VARSLC, SELCON and K2d among others (184, 185). In addition, an online server, DICHRWEB, has been developed and hosted in Birkbeck College, University of London, United Kingdom, which provides a platform for the deconvolution of CD data using algorithms, including those listed above, and a host of reference data sets (186, 187).

Like other spectroscopic techniques, CD has its limitations. In comparison to structural techniques such as X-ray crystallography and nuclear magnetic resonance (NMR), CD is a low resolution technique in which the spectrum reflects an average of the entire molecular populations in the sample. Specific solvents are required for optimum results since certain solvents (e.g. Tris and phosphate buffers) absorb significantly below 200 nm. These notwithstanding, CD is the more versatile and less cumbersome structural technique among the lot.
**CD units and conversions**

The CD signal is positive when L is absorbed to a greater extent than R, and negative when R is absorbed to a greater extent than L. In CD analysis, several units and conversions are employed to normalize data obtained from different protein systems and/or instruments, for easy comparison and interpretation. For example, the difference in absorption ($\Delta A$) is generally expressed in degrees of ellipticity ($\theta$), a common feature in the literature, using the following relationships:

$$\Delta A(\lambda) = A(\lambda)_L - A(\lambda)_R$$  \hspace{1cm} (3.3)

$$\theta = 32.982 \times \Delta A$$ \hspace{1cm} (3.4)

In practice, the observed ellipticities are of the order of 10 millidegrees. This is mainly because the $\Delta A$ detected for proteins is in the range of $10^{-4}$ to $10^{-6}$ absorbance units. The $\Delta A$ measured can be converted to molar circular dichroism $\Delta \varepsilon$ (M$^{-1}$cm$^{-1}$) which takes into account the molar concentration $c$ (M) of the sample being measured, as well as the pathlength $l$ (cm) of the optical cell.

$$\Delta \varepsilon = \Delta A / c \times l$$ \hspace{1cm} (3.5)

The molar ellipticity $[\theta]$ (deg.cm$^2$.dmol$^{-1}$ or deg.M$^{-1}$.m$^{-1}$) of the sample can then be calculated from $\Delta \varepsilon$ using the following relationship:

$$[\theta] = \Delta \varepsilon \times 3298.2$$ \hspace{1cm} (3.6)

Alternatively, $[\theta]$ can be calculated independently from the following equation:

$$[\theta] = 100 \times \theta / c \times l$$ \hspace{1cm} (3.7)
Presentation of CD data as ∆ε or [θ] allows the comparison of different samples irrespective of the concentration of the absorbing species or the pathlength of the optical cell used for the measurement.

Furthermore, the molar ellipticity can be reported for individual amino acid residues instead of an average of the bulk protein solution. This is achieved by converting the molar concentration (c) into mean residue concentration (c_{mr}) using the following relationship:

\[ c_{mr} = c \times N \]  

(3.8)

Where N is the number of amino acid residues in the protein. By replacing c with c_{mr} in equation 3.7, the resulting [θ] is termed mean residue ellipticity (MRE), which is commonly reported in the literature. In the event that the sequence of the protein sample is unknown, c_{mr} can be estimated using both the generic amino acid residue mean weight of 113 Da and the protein concentration c (gL^{-1}), in the following relationship:

\[ c_{mr} = \frac{c}{113} \]  

(3.9)

3.3.2 Application

A J-815 CD spectrometer (Jasco, Tokyo, Japan) was used for all the CD data reported in this work. The instrument is equipped with a peltier system, which controls both temperature and stirring speed. The light source is a compact, nitrogen-cooled, xenon arc lamp with a supplier reported wavelength range of 163 – 900 nm. Data were acquired in both the far-UV (190 – 240 nm) and near-UV (250 – 350 nm) regions of the electromagnetic spectrum.

The instrument software allows data acquisition at fixed wavelengths or a scan over a range of wavelengths. For a typical experimental setup, the set parameters were: scan range of 190 to 240 nm, data interval of 0.1 nm, bandwidth of 1.0 nm, response time of 4
s, and a scanning speed of 50 nm/min. The recorded spectra were an average of three scans. The background spectrum was used as baseline for data collection.

### 3.4 Atomic force microscopy

The atomic force microscope (AFM) has proven to be a versatile imaging tool for many biological systems, including proteins and polynucleotides, and is the preferred technique for 3D measurements on the nanometer scale. It allows for the investigation of morphological transitions that accompany protein aggregation and more recently, as a tool for quantitative measurements of biological interactions including hydrogen bonding and electrostatic forces. The common choice of AFM over other imaging techniques such as electron microscopy (EM) is due to its ability to directly monitor dynamic changes in the conformation, association, or functional state of individual biomolecules in aqueous environments that mimic their physiological state in situ (188). For example, AFM has been used to monitor amyloid fibril growth in vitro (189).

#### 3.4.1 Principles

The atomic force microscope exploits the deflections of a flexible pointed needle (cantilever), as it moves across an uneven surface, to give the topography (image) of that surface. The primary components of a typical AFM include a cantilever, light source, a position detector and a feedback mechanism to adjust the cantilever-to-sample height as it scans the sample surface. The cantilevers are typically microfabricated from silicon or silicon nitride to produce a pointed tip with radius of curvature at the nanometre scale. The cantilever is held in a tip holder and driven by a piezoelectric element. When the cantilever tip comes into contact with the sample surface, the forces of interaction between the sample and the tip induce a deflection of the cantilever; a phenomenon which can be explained by Hooke’s law.

\[ F = -kx \]  \hspace{1cm} (3.10)
where $x$ represents the distance between the deflected position and reference position, $k$ is the spring constant, and $F$ is the restoring force. Conventional AFMs employ a beam deflection system to monitor the position of the tip as it moves across the sample surface. Here, a laser beam is reflected from the back of the tip onto a position sensitive detector composed of an array of photodiodes. Changes in the reflected beam, resulting from tip deflections as the cantilever scans a sample surface, gives a lateral resolution or topology of the sample. A schematic diagram for the beam deflection system is shown in figure 3.4.

![Figure 3.4: A schematic representation of the beam deflection systems in atomic force imaging.](image)

The AFM can operate in a variety of modes which are generally categorized as contact or dynamic. In this work, intermittent contact mode, also referred to as tapping mode, was used to acquire images of protein aggregates. Here, the piezoelectric element drives a stiff cantilever to oscillate near its resonance frequency close to the sample surface. As the cantilever is scanned over the sample, part of the oscillation extends into a repulsive regime so that the tip intermittently touches (or taps) the surface. A feedback mechanism employs a piezoelectric actuator to control the cantilever-to-sample height in order to maintain the set cantilever oscillation amplitude. Detection of changes in the resonant
frequency or amplitude of the cantilever translates into the topology of the sample surface.

3.4.2 Application
An Asylum MFP 3D atomic force microscope (Santa Barbara, California) was used in this study. The instrument was mounted on a vibration isolation table to provide a stable surface during scanning. The cantilevers used were Silicon Budget sensors Tap300A1 ($f_0 = 270 – 300$ kHz, $k = 1.4 – 10$ N/m). Protein samples to be imaged were first applied to freshly cleaved mica, and then allowed 1–2 h to adsorb onto the mica surface whiles drying in a laminar flow cabinet. Excess protein solution was gently washed off the mica surface using a steady flow of Milli-Q water. The mica was then dried using nitrogen gas.

The AFM was set to tapping mode for image acquisition in air. For typical data collection, a low resolution scan was initially performed followed by a high resolution scan of areas of interest. Typical scan size was $10 \times 10 \mu m$ (low/high resolution) or $5 \times 5 \mu m$ (high resolution). Scan rates were between 0.8-1.0 Hz.

3.5 Analysis of fluorescence and CD data using phase diagrams
To detect conformational transitions as well as intermediate states involved in protein denaturation, spectral data from fluorescence and CD experiments were further analysed using transition phase diagrams. In brief, the phase diagram is a plot of the spectral intensities of two arbitrary wavelengths, $I_{\lambda 1}$ vs. $I_{\lambda 2}$, for a protein undergoing conformational transitions. Interpretation of structural transitions is based on the linearity or otherwise of the relationship $I_{\lambda 1} = f[I_{\lambda 2}]$ (39). A single linear relationship on the plot suggests an all-or-none transition between two different conformations, while non-linearity (or multiple linear segments) suggests a sequence of structural transitions where each linear segment indicates individual all-or-none conformational transitions (190). It is noteworthy that phase plots are more informative when $\lambda_1$ and $\lambda_2$ are on different slopes of the spectrum (191).
3.6 Couette cell for uniform flow studies

A custom-built optical-quartz-cell with Couette geometry (a stationary outer cylinder and a rotating inner cylinder) was used for all shear flow experiments that required a uniform flow field. The stationary outer cylinder is a quartz block with a cylindrical hole bored through the centre to accommodate the mobile inner cylinder, a quartz rod. The gap between the inner and outer cylinders is 0.0175 cm. The top and bottom ends of the cell were capped with teflon ferrules to hold samples in place and also facilitate the precise centring of the inner cylinder. The inner cylinder is attached to an electric motor assembly that drives it to achieve varying shear rates under laminar flow. For a Newtonian fluid, the shear rate is effectively uniform across the narrow gap between the two cylinders. The rotational speeds were measured by a frequency counter attached to the motor and the shear rate (\( \dot{\gamma} \)) was calculated using the following equation:

\[
\dot{\gamma} = \frac{2\Omega}{(1-(R_1/R_2)^2)}
\]  

(3.10)

\( \Omega \) = angular frequency

\( R_1 \) = radius of inner cylinder

\( R_2 \) = radius of outer cylinder

The Couette cell was used for both fluorescence and circular dichroism measurements. Here, the cell was mounted in a holder and aligned in either instrument such that the cell was in line with both the beam originating from the light-source and the detector. Fluorescence data were collected at 90° angles to the beam path whereas the linear detection system of CD, typical of absorption spectroscopy, was used for data collection. A schematic diagram of the Couette cell and a photograph of the cell mounted in a holder are shown in figures 3.5 and 3.6.
Figure 3.5: Schematic depicting the shear device, and Couette flow profile (inset). The flow is in the \( x \)-direction whereas the velocity gradient is in the \( y \)-direction.

Figure 3.6: A photograph of the Couette rheofluorescence cell mounted in the cell holder with supporting base platform for fluorescence studies. A modified platform was used for the CD studies.
4. Tyrosine Autofluorescence as a Measure of Bovine Insulin Fibrillation

4.1 Introduction

The traditional approach to investigating the partial unfolding and fibrillation of insulin, and proteins at large, has involved use of the dyes 1-anilinonaphthalene-8-sulphonic acid (ANS) and Thioflavin T (ThT) respectively. Although these dyes have proven instrumental in our understanding of protein aggregation, their mode of action and possible contribution to the aggregation process has yet to be fully understood. For this reason, it is essential to apply an array of complementary techniques when studying the complexities of structural changes associated with protein aggregation.

Intrinsic protein fluorescence is a sensitive technique that has been exploited in studies of the structural, physicochemical, and functional properties of proteins (192). Proteins derive their intrinsic fluorescence from the chromophores Phe, Tyr and Trp (193). Bovine 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 non-aromatic absorption attributable to cystine, histidine or the peptide bond (194). A sigmoid curve is observed in Tyr fluorescence emission during insulin aggregation. Hence, the focus of this work was to investigate the physical and chemical transformations associated with insulin aggregation by means of its intrinsic fluorescence.

It is noteworthy that obtaining such critical information from the protein itself is complementary to existing aggregation probes, and affords the advantage of directly examining structural changes occurring at the molecular level. Multiple probes were employed in order to better understand the molecular dynamics, as indicated by intrinsic Tyr fluorescence, involved in this process. Protein denaturation results in a marked uniformity in its intrinsic fluorescence characteristics (195), and that is what was expected for insulin under the experimental conditions used in this study.
4.2 Materials and methods

*Materials:* Bovine insulin and Thioflavin T (ThT) were obtained from Sigma (St. Louis, MO). The hydrophobic probe 1-anilinonaphthalene-8-sulphonic acid (ANS) was purchased from Aldrich (Milwaukee, WI). All other reagents used were of analytical grade.

*Sample preparation:* Insulin solutions were prepared by dissolving the peptide directly in 0.1% (v/v) HCl (pH 1.9) to give a concentration of ~0.2 mg/mL. The final insulin concentration was calculated from UV absorption at 280 nm, applying a molar extinction coefficient of 5.53 mM$^{-1}$cm$^{-1}$. For insulin/dye samples, 1mM stocks of ThT and ANS were prepared by dissolving each dye in double distilled water, followed by filtration through a 0.22 µm filter paper. The final dye concentration in insulin samples was 50µM.

*Fluorescence spectroscopy:* Fluorescence studies were conducted using the Varian Cary Eclipse Fluorescence spectrophotometer described in chapter 3. All measurements were obtained using a 1 cm pathlength quartz cuvette equipped with a 5mm magnetic stirrer bar (spin rate ~120 rpm). The instrument was set to collect data at multiple wavelengths simultaneously in order to monitor both intrinsic (Tyr) and extrinsic (ThT/ANS) fluorescent probes during insulin aggregation. For insulin fibrillation, 3 mL of the reaction sample was incubated at 60°C over a period of 22 h. Intrinsic insulin fluorescence was monitored via Tyr molecules using an excitation wavelength of 276 nm and emission measured at 303/305 nm (kinetic studies) or 280 – 500 nm (interval scans). Tyrosinate and dityrosine fluorescence were measured using excitation/emission wavelengths of 295/345 nm and 315/410 nm respectively. ThT fluorescence emission was measured at 482 nm on excitation at 450 nm whereas ANS fluorescence was probed using excitation and emission wavelengths of 350 nm and 460 nm respectively.

*Light scattering:* The fluorometer described above was set to measure real-time light scattering alongside the fluorescence parameters during bovine insulin aggregation. The excitation wavelength was set at 600 nm, away from the absorption regions of the chromophores present, and scattered light measured at 605 nm (shoulder of excitation
peak). The data was collected at 5 minute intervals using excitation and emission slit widths of 10 nm unless stated otherwise.

*Data analysis:* Fluorescence and CD data were further analysed using phase diagrams as described in chapter 3 (39, 191).

*Atomic force microscopy (AFM):* Imaging of insulin fibrils was done using the MFP 3D AFM (Asylum, Santa Barbara, CA). The data was acquired in air using silicon nitride probes in intermittent contact mode.

*Secondary structure of insulin fibrils:* Far-UV circular dichroism (CD) spectra of fibrillated insulin samples were recorded at room temperature using the J-815 CD spectrometer (Jasco, Tokyo, Japan). A 100 µL aliquot of insulin preparations incubated at 60°C with stirring for 22 h were scanned in a 0.2 cm pathlength quartz cell from 195 to 240 nm. Solvent spectrum was used as baseline for data collection. The final spectrum of all samples was an average of three independent scans.

*Thermal denaturation/renaturation experiments:* The effect of temperature on insulin structure was studied by fluorescence and CD spectroscopy; conducted over a temperature range of 10°C – 95°C at 5°C intervals. The concentration of insulin used was ~0.2 mg/mL in all experiments. For intrinsic fluorescence studies, emission scans of Tyr molecules were recorded from 280 – 500 nm, in a 1 cm pathlength quartz cell, using an excitation wavelength of 276 nm. CD spectra for both near- and far-UV were recorded using the J-815 CD spectrometer (Jasco, Tokyo, Japan). Insulin samples (200 µL) were scanned in a 0.2 cm pathlength cell from 195 to 340 nm using a data interval of 0.1 nm, a bandwidth of 1.0 nm, response time of 4 s, and a scanning speed of 50 nm/min. Both fluorescence and CD data were collected after a delay time of 5 min at each temperature.

**4.3 Results**
The kinetics of insulin denaturation, aggregation and subsequent fibrillation as detected by multiple parameters were normalized to unity and shown in figure 4.1. The amyloid
probe ThT was employed to detect fibril development; ANS to detect solvent-exposed hydrophobic groups during insulin unfolding and aggregation; light scattering to detect the increase in particle size (aggregates) in solution; and intrinsic Tyr fluorescence to study the physicochemical changes associated with insulin fibrillation. Reaction samples were incubated at 60°C and stirred continuously at ~120 rpm for 22 h after which ThT assay, AFM images, and CD spectra confirmed the presence of amyloid fibrils.

![Graph showing kinetic profile of ThT](image)

Figure 4.1: Kinetics of insulin fibrillation detected by multiple parameters. Insulin denaturation and subsequent fibrillation was detected by changes in the fluorescence emission of ThT (△), Tyr (◇), ANS (□) and light scattering (—).

**Kinetics of insulin aggregation/fibrillation**

The kinetic profile of ThT, shown in figure 4.1, exhibited a typical sigmoid behaviour indicative of a nucleation dependent fibril assembly proceeding in 3 phases (161): (i) a lag phase, during which a fibril competent nucleus evolves; (ii) a growth phase, with rapid polymerization of amyloidogenic nuclei into fibrils; and (iii) fibril maturation at the equilibrium phase. The validity of this mechanism of insulin fibrillation is supported by data obtained from complementary studies described below.
From figure 4.1, a dramatic increase in ANS fluorescence and quenching of Tyr fluorescence were observed to occur simultaneously at ~2 h of incubation, both reaching equilibrium after 8 h. Both profiles were obtained simultaneously from an insulin-ANS sample. The enhancement of ANS fluorescence emission at 460 nm indicates the presence of solvent-exposed hydrophobic regions, originating from partially folded intermediates, in solution. The concurrent decrease in Tyr fluorescence suggests that the Tyr residues were within hydrophobic pockets in the protein matrix, and became accessible to effective quenchers upon exposure to the aqueous environment. Indeed Tyr-A\textsuperscript{14}, Tyr-B\textsuperscript{16} and Tyr-B\textsuperscript{26} are resident in the two hydrophobic faces of the insulin monomer that associate to form dimers (196); and the emission intensity at 305 nm is an average of the parametric changes affecting the microenvironment of all Tyr residues. It is worth noting that the lag times for these changes terminated ~3 h before the onset of ThT fluorescence enhancement at 482 nm, which shows that partially folded intermediates indeed precede insulin fibrillation.

The light scattering profile shows the gradual development of large aggregates ( oligomers) in solution from the onset of incubation, with a dramatic increase occurring after ~3 h. This clearly shows that the early insulin aggregates formed, although light-scattering, escape detection by ThT. The data also reveal that the partially folded intermediates, which begin to evolve after ~2 h incubation, associate rapidly into these oligomers from which fibrillar aggregates develop. The occasional spikes experienced in the kinetic trace are attributable to the polydispersed nature of insulin aggregates formed during fibrillation (18, 197).

The observed intrinsic Tyr fluorescence profile during insulin fibrillation, relative to those of ThT and ANS, informed the design of an experimental approach to study insulin fibrillation via its intrinsic fluorescence.

*Insulin fibril morphology and secondary structure*

AFM height traces of insulin fibrils formed in the presence or absence of dyes are shown in figures 4.2a-c. A dense network of fibrils is observed in all cases, consistent with that
reported elsewhere (159). The crowded nature of the fibril network made it difficult to
measure heights of individual fibrils relative to the mica surface. However, it is clear that
fibrils formed in the absence of a dye were thinner relative to those formed in the
presence of either ThT or ANS. This observation suggests variations in fibril morphology
but is not conclusive. The images were acquired in the order in which they are presented,
which prevents the possible introduction of an artefact, for example, tip broadening of
measured lateral dimensions. Complementary studies using electron microscopy provided
similar results.

Figure 4.2: Morphology and secondary structure of fibrillated insulin. (a-c) AFM height
trace images comparing insulin fibrils formed in the presence of ThT (a) and ANS (b),
and in the absence of a dye (c). The images show variations in fibril morphology
depending on the dye present. Scale bars, 1µm. (Lower panel) Far-UV spectra of insulin
fibrils at pH 1.9. The figure shows a plot of the mean residue ellipticity (MRE) as a
function of wavelength. The spectra represent fibrils formed in the presence of ThT (▲)
and ANS (○), and in the absence of a dye (- -). The data were collected at ambient
temperature, and each trace is an average of three independent scans.
The far-UV CD spectra of insulin samples incubated for 22 h at 60°C in the presence and absence of ThT/ANS are shown in figure 4.2 (lower panel). All three spectra are characteristic of β-sheet secondary structure, but with noticeable differences in trace and minimum ellipticity, depending on the dye present. The spectra obtained for dye-free insulin and insulin-ThT solutions both show minimum ellipticity at 220 nm, whereas that obtained for the insulin-ANS sample has its peak negative intensity at 222 nm. These minima show a red shift from the characteristic β-sheet signature which occurs between 215 nm and 218 nm. The occurrence of this feature can be ascribed to residual helical structure in fibrillar insulin, possibly originating from the B-chain helix (191). It is also possible that the observed differences are a consequence of insulin-dye interactions, leading to polymorphisms in the resulting fibril structure.

*Monitoring insulin fibrillation via Tyr fluorescence*

At ambient temperature, intrinsic Tyr fluorescence of insulin preparations showed an emission $\lambda_{\text{max}}$ at 305 nm with two excitation peaks at 276 nm and 227 nm. This indicates that the freshly prepared reaction solutions did not contain hydrogen-bonded Tyr residues.

Two approaches were used to monitor changes in Tyr fluorescence for a dye-free insulin sample incubated under conditions favouring fibrillation. The aim was to explain the Tyr fluorescence quenching observed during insulin aggregation by probing the possible chemical modifications the fluorophore may access. The first method was to conduct interval scans of the emission spectrum during insulin aggregation (figure 4.3a). The insulin sample was excited at 276 nm, with emission scans from 280 - 500 nm at 5-min intervals (1-h interval scans are shown for clarity). The scans show a decrease in Tyr fluorescence emission intensity during insulin fibrillation. This becomes apparent on plotting the emission maxima at 305 nm against time (figure 4.3a, *inset*), which gives a sigmoid curve similar to that of the Tyr fluorescence profile in figure 4.1. The data was further analysed using phase diagrams, which have proven versatile in detecting intermediate states of a protein undergoing structural transition from an initial to final state (198).
Figure 4.3: Insulin denaturation/fibrillation detected by Tyr fluorescence. (a) Fluorescence emission spectra of tyrosine during insulin aggregation. The fibrillating insulin sample (0.2 mg/mL) was excited at 276 nm with emission scans from 280-400 nm. The figure shows a consistent decrease in intensity of the emission $\lambda_{\text{max}}$ (305 nm) after $\sim 2$ h of incubation, reaching equilibrium after 12 h. (Inset) A sigmoid is observed on plotting the emission intensity at 305 nm against time. (b) Fluorescence phase diagram obtained by plotting $I_{330\text{nm}}$ against $I_{305\text{nm}}$ shows the multiple structural transitions accompanying insulin aggregation.

A plot of the emission intensity $I_{330\text{nm}}$ against $I_{305\text{nm}}$ from the fluorescence data (figure 4.3b) show three linear segments spanning 0–3 h, 3–12 h, and 12–22 h. Each linear segment describes a distinct structural transition, revealing the existence of four structurally unique species during insulin fibrillation. Using information gathered from
The second approach involved monitoring Tyr fluorescence at fixed wavelengths. This allowed simultaneous studies of changes in Tyr fluorescence (excitation 276 nm, emission 305 nm) and the possible formation of tyrosinate (excitation 295 nm, emission 345 nm) and/or dityrosine (excitation 315 nm, emission 410 nm) during insulin aggregation. The intrinsic Tyr fluorescence kinetic profile showed the familiar sigmoid curve (figure 4.4a). There was no significant change in the emission intensity at the wavelengths associated with tyrosinate and dityrosine over the 22-h period (see figure 4.4). The phenol group of Tyr has a pKa of 9.8 and 4.2 for the ground and excited states respectively (201). At pH 1.9, the phenol group of Tyr and its possible proton acceptors, including Glu and Asp residues, are fully protonated, which explains the absence of tyrosinate, and hence dityrosine, during insulin aggregation.

It is noteworthy that the position and shape of the fluorescence emission spectrum of Tyr in fibrillated insulin solutions exhibited the same peaks observed in a freshly prepared sample, though considerably diminished in intensity. This reaffirmed that Tyr residues present in the insulin fibrils were non-hydrogen-bonded. Indeed, early studies by Waugh showed that the conversion of native insulin molecules into fibrillar aggregates did not involve chemical modification (202). Recent studies using α-synuclein at pH 7.4 found that whereas there was no evidence of dityrosine formation during fibrillation, this covalent linkage was observed in the soluble aggregates present after fibrillation was complete (203).
Figure 4.4: Structural changes during insulin aggregation as detected by tyrosine fluorescence kinetics. The emission intensities of Tyr (panel a), tyrosinate (panel b) and dityrosine (panel c) were probed during insulin aggregation. The error bars show ± standard deviations.
Figure 4.5: Thermal denaturation of bovine insulin (0.2 mg/mL) as detected by fluorescence and CD spectroscopy. (a) Decrease in Tyr emission intensity at 305 nm and loss of secondary structure on heat denaturation (open triangles). The reverse is observed on renaturation (open circles). The temperature range was between 10°C and 95°C, and data were collected at 5°C intervals. Phase diagrams obtained from fluorescence (b) and CD (c) data reveal the existence of structural intermediates on thermal denaturation of insulin. Error bars indicate the ± standard deviations of duplicates of each experiment.
Temperature effect on bovine insulin

Thermal denaturation and renaturation of bovine insulin and associated changes in its structure were studied by fluorescence and CD spectroscopy; conducted independently but under comparable experimental conditions. The aim was to further understand the sensitivity and, hence, applicability of intrinsic Tyr fluorescence in studying conformational and structural changes in bovine insulin during aggregation. CD spectra in the far-ultraviolet wavelength range (typically from ~190 to 250 nm) provide useful information on the secondary structure of proteins(186), whereas intrinsic protein fluorescence (in this case Tyr fluorescence) provides basic information on the dynamics of structural reorganization in protein molecules (198). At ambient temperature, the far-UV spectrum of native insulin showed two negative peak intensities at 209 nm and 222 nm, typical of an α-helical secondary structure, and these intensities have been used in studying insulin denaturation (204).

Thermal unfolding of the helical segments of insulin was monitored using the change in ellipticity at 222 nm, whereas Tyr fluorescence emission was monitored from 280 – 500 nm on excitation at 276 nm (the intensity at $\lambda_{\text{max}}$ 303 nm is shown) (figure 4.5a). The far-UV spectrum show that temperature elevation results in a significant decrease in the negative intensity at 222 nm, indicating a loss in helical structure (205). Such conformational changes obviously would affect the local environment, especially of the three Tyr residues resident in the helical regions of bovine insulin (159). True to this, the Tyr emission intensity at 303 nm showed a 73% decrease as a function of temperature on heating from 10°C to 95°C. It has been established that the emission $\lambda_{\text{max}}$ of Tyr is independent of temperature(39), and the location of non-hydrogen-bonded Tyr in a protein’s hydrophobic interior or α-helical segment enhances its quantum yield (or intensity) (206). Therefore, the observed decrease in Tyr fluorescence intensity is attributable to effective Tyr–quencher interactions facilitated by: (i) heat-induced structural disorder of the native state via perturbation of intra-/inter-molecular interactions (including hydrogen bonding, van der Waals, hydrophobic and electrostatic interactions); and (ii) enhanced dynamic mobility of the entire protein matrix, dictated by solvent viscosity (195).
Both fluorescence and CD profiles show that the major transition occurred between 25°C and 80°C. It is important to note that the fluorescence profile also show that the transitions involved are completely reversible, as the emission intensities at various temperatures were restored on renaturation. However, the CD profile on renaturation show ~13% loss in the native helical content (with reference to the intensities at 10°C), which suggests that some molecular interactions were permanently damaged on denaturation hence preventing the complete refolding of insulin on cooling.

Further analysis of the fluorescence and CD data using phase plots are shown in figures 4.5b and 4.5c respectively. Fluorescence phase plots were obtained by plotting $I_{330\text{nm}}$ against $I_{303\text{nm}}$, whereas the negative ellipticities at $\theta_{209\text{nm}}$ and $\theta_{222\text{nm}}$ were used to generate CD phase plots. The peak wavelengths from the CD spectrum were chosen, as these have been successfully applied in building phase plots to distinguish structural transitions during insulin fibrillation (191). Both plots show three linear segments, indicative of the existence of two intermediate states during the transition from native to temporarily denatured insulin. A closer analysis of the plots show that whereas the linear segments of the fluorescence data span 10–25°C, 30–55°C and 55–95°C, those of the CD data span 10–45°C, 45–70°C, and 70–95°C. The difference in the temperature range of the linear segments for the two probes indicates that they differ in their sensitivity to structural changes in native insulin, and hence can be applied as complementary techniques in studying insulin denaturation.

The initial transition (10–25°C) in the fluorescence plot may be attributed to the decrease in viscosity of the protein matrix enhancing intramolecular collisions between excited Tyr side chains and neighbouring quenchers. The CD plot shows no major conformational change within this temperature range. The second fluorescence transition (30–55°C) corresponds to the dissociation of dimers into monomers and the emergence of the so-called heat-induced molten insulin globule, which is the major species in solution at ~55°C (207). It is noteworthy that this is the first major conformational transition detected by CD (at ~45°C) reaching a maximum at ~70°C. Indeed, differential scanning calorimetric scans of bovine insulin (pH 1.9) showed an endothermic transition midpoint
at 60°C (208). Therefore, transitions beyond 55°C can be ascribed to thermal unfolding of monomeric insulin into an unstructured, aggregation-prone intermediate.

It is worth noting that the tertiary/quaternary structure of bovine insulin, monitored at θ_{278nm} (typical Tyr signature), showed complex structural transitions occurring at temperatures similar to that observed in the fluorescence phase plots. However, the data were not considered in detail as the peak negative intensity at 278 nm was weak (near zero), indicating a lack of native tertiary interactions. This observation is ascribed to the low insulin concentration used in this study. This is consistent with the notion that insulin adopts a less rigid structure at low concentrations, with highly mobile aromatic side chains that show weak intensities (159). Disulfide bonds, three of which are present in insulin, are known to contribute significantly to the absorption in the near-UV region, which further complicates the interpretation of spectral data obtained from that region (180, 181).

**4.4 Discussion**

The use of fluorescent probes has found wide applications in studies of the structure and dynamics of proteins (39). *In vitro* studies of protein aggregation have employed the histological dye Thioflavin T to detect amyloid fibrils, and the hydrophobic dye ANS to establish the existence of partially folded intermediates on the pathway to protein fibrillation (209, 210). It is interesting to note that structural changes in proteins can be detected via intrinsic protein fluorescence (39); hence, this feature can be applied (in addition to other techniques) 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. In order 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.
Quenching of Tyr fluorescence during insulin aggregation

There are several possible catalysts for the observed Tyr fluorescence quenching preceding insulin fibrillation: (i) protolysis of the phenol group of Tyr when it interacts with solvent molecules and/or amino acid side chains, which can act as proton donors or acceptors or as hydrogen bonding partners; (ii) resonance energy transfer from Tyr to Tyr, Tyr to tyrosinate, Tyr to ANS, or Tyr to ThT; (iii) interactions between the excited chromophore and hydrated peptide carbonyl groups; or (iv) interactions between the excited chromophore and cystine groups (211). The occurrence of any of these mechanisms is dependent on the local environment of both the ground and singlet excited states of Tyr residues in the protein matrix, including their proximity and interactions with quenchers.

The ionisable groups in bovine insulin are fully protonated at pH 1.9. Since excited Tyr residues have a nominal pKa of 4.2, the possible deprotonation of the hydroxyl group and subsequent formation of tyrosinate or Tyr-carboxylate hydrogen bonds is theoretically impractical. The results from figure 4.3 show that the emission $\lambda_{\text{max}}$ of Tyr is constant at 305 nm, although considerably diminished in intensity, even after insulin fibrillation. Further supporting this finding, the kinetic experiments in figure 4.4 discounted the likelihood of transient hydrogen bonds between Tyr residues and solvent molecules, the peptide carbonyl, or neighbouring carboxyl groups. This was expected as the rate of depopulation of the excited state by emission occurs much faster than depopulation by excited-state protolysis (201). Taken together, the observed Tyr fluorescence quenching during insulin aggregation is unlikely to be due to protolysis of the phenolic chromophore in the ground or excited states.

The presence of multiple Tyr and Phe residues in bovine insulin raises the possibility of resonance energy transfer from Phe to Tyr and between adjacent Tyr residues. However, the 276-nm excitation wavelength used in this study would minimize the amount of light absorbed by Phe residues, because these residues show maximum absorption at 258 nm. The radii of globular proteins are reported to range between 15 Å and 30 Å, which is within acceptable limits for efficient energy transfer between donor and acceptor
molecules (212). It is anticipated, therefore, that the average distance between Tyr groups in insulin, given as 16.57 Å, will permit efficient intertyrosine resonance energy transfer (194). Indeed, fluorescence-polarisation measurements of insulin have established the occurrence of intertyrosine energy transfer (213). It is also expected that Phe residues will contribute to the overall quantum yield of Tyr fluorescence. Resonance energy transfer between Tyr and ANS has been reported (214), and it has been observed (in this work) that on excitation at 276 nm, the emission spectrum of Tyr in insulin overlaps with the excitation spectrum of unbound ThT. Taken together, it can be deduced that the initial structural reorganization preceding insulin fibrillation brings aromatic residues into proximity, hence facilitating energy transfer between donor and acceptor molecules within the protein matrix and in solution. A probable consequence of this dynamic association is the observed quenching of Tyr fluorescence.

Insulin contains three native disulfide bonds that have been established as strong quenchers of intrinsic fluorescence (39). These covalent bonds are known to be highly hydrophobic and, hence, shielded from the aqueous environment in native insulin (160, 215-217). Tyr is partially hydrophobic, and three out of the four Tyr residues in bovine insulin are located in hydrophobic pockets and involved in hydrophobic interactions resulting in various association states of the protein (166). For this reason, interactions between Tyr residues and disulfide bonds can only occur upon exposure of hydrophobic groups to the solvent. The results show that ANS fluorescence enhancement, which indicates the presence of solvent-exposed hydrophobic regions, coincides with Tyr fluorescence quenching (figure 4.1). This is a clear indication that the ANS binding sites are in proximity to most of the Tyr residues (210), and that exposure of these hydrophobic regions makes Tyr accessible to quenchers. It should be noted that the disulfide bonds, which are strong quenchers of intrinsic protein fluorescence, also become solvent-accessible upon insulin denaturation, and their interactions with excited Tyr residues is therefore greatly enhanced.

The results show that Tyr fluorescence quenching persists in the absence of dyes (figure 4.3a and 4.4a). This suggests that its occurrence is predominantly a result of intra-/inter-
molecular singlet-singlet energy transfer between adjacent Tyr residues (213) and, most probably, effective contacts between excited singlet Tyr residues and cystinyl side chains (39). The frequency of these interactions is determined by the heat- and agitation-induced fluidity of the protein matrix resulting from the disruption of hydrophobic pockets and other stabilizing forces that maintain the native protein structure (178). This is supported by the temperature dependence of tyrosine fluorescence, giving ample evidence that the contact quenching mechanism is collisional and not static. The mechanism of Tyr fluorescence quenching by cystine is debated. The suggested possibilities include deactivation of the excited state molecules via internal conversion or enhanced intersystem crossing to the triplet state (176).

In summary, the start of Tyr fluorescence quenching signifies the emergence of solvent-exposed hydrophobic groups, hence partially folded intermediates, in solution.

**Insulin fibrillation detected via Tyr fluorescence**

In a 0.1% HCl solution (pH 1.9) at room temperature, insulin is predominantly dimeric, maintaining its native fold (159). At 60°C, however, the equilibrium favours the monomeric state and protein denaturation is known to free phenolic groups bound in the native protein. From figures 4.1 and 4.3a (inset), the initial 2 h preceding Tyr fluorescence quenching is the time taken for the dissociation of existing dimers into monomers and disruption of the stabilizing forces of native insulin monomers. The result is thermal and agitation-induced denaturation of monomeric insulin and consequent exposure of hydrophobic regions to the aqueous environment. The start of Tyr fluorescence quenching at ~2 h is indicative of the emergence of a new structural species, a partially unfolded intermediate, in solution, a conclusion that is buttressed by the results of ANS experiments. This is consistent with reports that partially folded intermediates precede insulin fibrillation (30). The light scattering profile in figure 4.1 shows that this new structural species associates rapidly into large (oligomeric) aggregates. The Tyr fluorescence phase plot (fig. 4.3b) equally detects these aggregates evolving at ~3 h, reaching a maximum concentration at ~12 h. Mass spectrometry studies on amyloidogenic insulin samples incubated at high temperatures revealed that the
dissociation of soluble higher oligomers (e.g., dimers) is followed by the formation of high-molecular-weight aggregates, from which fibrils evolve (197).

The emergence of these oligomeric aggregates is attributable to an interplay of electrostatic and hydrophobic interactions that allow self-association of denatured monomers. For example, the chloride ions present in the solvent screen the net positive charge on native insulin at pH 1.9, allowing the coalescence of monomers, via hydrophobic interactions between partially unfolded intermediates, to form a fibril-competent nucleus. This effect of chloride ions is purported to contribute to the precipitation of insulin into a dense network of fibrils (159). The absence of hydrogen-bonded Tyr residues, coupled with the insulin-solvent charge interactions at pH 1.9, supports the hypothesis that the attractive force driving the association of partially unfolded monomers into oligomers at 60°C is hydrophobic in nature. ThT fluorescence begins to increase partway (~5 h, figure 4.1) through the formation of these intermediates, which implies that the oligomers, so formed, begin to polymerise into fibrillar aggregates, which are the dominant structural species at 22 h. The saturation of Tyr fluorescence quenching at ~12 h (figure 4.3a inset) indicates that the majority of Tyr residues, hence hydrophobic regions, have become solvent-exposed at that time. ThT fluorescence reaches equilibrium after 18 h, which suggest that most of the fibril-competent nuclei are consumed into mature fibrils at that time. Based on these results, it is clear that the major structural changes during insulin aggregation can be detected via Tyr fluorescence.

The sequence of structural changes accompanying insulin fibrillation and probes sensitive to each structural transition, as observed in this study, is summarised in figure 4.6. It is noteworthy that the phase plots obtained from intrinsic Tyr fluorescence are sensitive to these structural changes (figure 4.3).
Biophysical differences in insulin fibrils formed in the presence or absence of dyes

The AFM images show that the physical appearance of insulin fibrils differs depending on the dye present. Insulin fibrils formed in the absence of ANS or ThT were finer, whereas fibrils formed in the presence of those dyes appeared thicker, indicating a higher degree of association. In the case of ANS, the sulphonate group of the dye is negatively charged at pH 1.9, and in complement with chloride ions may screen the net positive charge on insulin, leading to a more significant precipitation of fibrils. ThT, on the other hand, is known to bind along cavities running parallel to insulin fibrils, hence contributing to the overall fibril structure. Indeed, X-ray diffraction studies have confirmed increased morphological variation in insulin fibrils formed in the presence of ThT compared to those formed in the absence of the dye (209). Such structural discrepancies, in addition to the residual α-helical structure known to exist in fibrillated insulin, may account for the differences in CD spectra for fibrils formed in the presence
or absence of ThT/ANS. It is also likely that the dyes, aside from influencing the fibril structure, also perturb the electron cloud of insulin amide chromophores responsible for the far-UV CD signal, resulting in the observed differences in intensity and peak position (218). It is noteworthy that the techniques applied here do not allow a firm conclusion to be drawn from these observations. Further analysis using techniques such as X-ray diffraction is necessary for concrete comparison of fibrils formed under the different conditions.

Relevance of monitoring insulin aggregation via its intrinsic fluorescence
The nonintrusive but sensitive nature of intrinsic protein fluorescence makes it a versatile tool used in concert with other protein aggregation probes (especially extrinsic probes) to gain additional information on the structural changes associated with insulin deformation and aggregation. The merit in obtaining such critical information from the protein itself cannot be overemphasised. For example, it affords the advantage of directly monitoring changes occurring in the peptide-hormone at the molecular level during aggregation, giving concrete detail on the early events preceding fibrillation. This approach is suitable for real-time fluorescence studies conducted in situ. It is noteworthy that the light-scattering technique used in this study only provides qualitative evidence of the association of deformed insulin monomers into larger aggregates. However, its sensitivity to the size increase of insulin aggregates in solution makes it a suitable complementary probe in monitoring protein aggregation.

4.5 Conclusion
Intrinsic Tyr fluorescence can be used to resolve the complex structural modifications associated with insulin fibrillation. The observed Tyr fluorescence quenching during insulin aggregation is attributable to energy transfer between Tyr residues and acceptor molecules (including adjacent Tyr residues, ThT and ANS) and the likely repetitive collisions between exposed Tyr residues and indole fluorescence quenchers such as the disulfide bonds present in native insulin. There was no evidence of tyrosinate or dityrosine formation during insulin aggregation.
5. Amyloid-β Fibrillation in Heterogeneous Flow

5.1 Introduction

Alzheimer’s disease (AD) is a common form of senile dementia resulting from protein conformational disorders, and has been recognized in medicine since 1906 (219). Typical pathological features of the disease include brain amyloid plaques composed of both proteinaceous and nonproteinaceous elements. Since its diagnosis, AD has been a major research priority in medical science, although a complete understanding of the aetiology of the disease and the development of therapeutic interventions has yet to be realised.

Fibrillar aggregates of Aβ peptide are the primary constituents of amyloid plaques in Alzheimer’s disease (156). To date, the physiological conditions and mechanisms that trigger Aβ aggregation and deposition as brain-amyloid plaque has yet to be fully understood. It has been found that the amyloidogenesis of a peptide/polypeptide chain is dependent on a number of intrinsic properties which include high hydrophobicity, high β-sheet propensity, and a low net charge (109). Amyloid-β has a hydrophobic C-terminal rich in β-strands(155), and an overall net charge of negative three at neutral pH (220).

It has been suggested that the strong aetiological association of Aβ aggregation and AD may be a result of assemblies formed at the early stages of Aβ oligomerisation (29). Accumulating evidence suggest that AD is a vascular disorder associated with deteriorations in microcirculation in the brain (221, 222). It is purported that such changes in circulation, accompanied by changes in the local shear stresses of the vascular system, may perturb the peptide’s native structure, and could therefore be a catalyst for Aβ fibrillogenesis. Since Aβ peptides found in the bloodstream are predisposed to varying shear stresses during transportation in microcirculation (223), it is important to investigate the effect of fluid forces, hence haemodynamic stress, on the native conformation of soluble Aβ peptides.
It is interesting to note that protein aggregation, \textit{in vitro}, is enhanced by mechanical agitation (heterogeneous flow) in the form of stirring, shaking, rotation, sonication and ultrasonication (47, 53, 54, 109, 111). The hydrodynamic stress resulting from agitation perturbs the noncovalent stabilizing forces, primarily hydrophobic and electrostatic interactions, of the functional native protein structure, promoting protein misfolding and subsequent aggregation (9). It is thus not surprising that agitation is a common feature of laboratory protocols employed in studies of protein aggregation. In most cases, it is applied in concert with other destabilizing conditions (e.g., temperature and/or pH) to promote fibril formation. Indeed, agitation has been shown to be a critical factor in Aβ\textsubscript{40}(224), whey protein(225) and bovine insulin fibril formation (15).

Aβ aggregation, \textit{in vitro}, involves a three step process: (i) partial folding of monomers; (ii) nucleation of monomers into fibril-competent molten oligomers; and (iii) rapid polymerization of oligomeric aggregates into mature fibrils (226). The nucleation phase is considered rate-limiting in Aβ fibril assembly (47). It involves the partial folding and coalescence of several Aβ monomers to form a critical nucleus, which serves as a scaffold for fibril assembly. Both nucleation and fibril assembly are facilitated by electrostatic and hydrophobic interactions (15). Circular dichroism, nuclear magnetic resonance, and FTIR analysis of this kinetic mechanism show a conformational transition of the Aβ peptide from an initial random coil structure, through an α-helical intermediate species, to a characteristic β-sheet structure ubiquitous in mature amyloid fibrils (29, 32, 227).

Although agitation is widely used to induce and accelerate fibril formation, little is known of the mechanism involved. That is, whether it affects the various phases (folding/unfolding, nucleation, and polymerisation) during amyloid fibril assembly. Considering the frantic search into unearthing the molecular mechanism underlying amyloid fibril assembly, it is imperative to appreciate the contributions of physical factors, especially mechanical agitation, which forms part of laboratory protocols. The work reported here is aimed at examining the overall kinetics of Aβ\textsubscript{40} fibrillation, under heterogeneous flow conditions generated via stirring.
5.2 Materials and methods

Materials: Monomeric Aβ_{40} was purchased from Keck laboratories, Yale University, and used without further purification. Thioflavin T was obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

Aβ_{40} sample preparation: Dilute solutions of Aβ_{40} were prepared by dissolving the peptide directly into phosphate buffer, pH 7.4, and stored at 4°C for ~16 h to allow for complete dissolution and equilibration. The samples were then diluted with a 1 mM stock of Thioflavin T (prepared in phosphate buffer) to give a final peptide and ThT concentration of 0.2 mg/ml and 50 µM respectively.

Fluorescence measurements: Fluorescence data were collected using the Cary Eclipse fluorometer. The reaction solutions were incubated at 37°C in a 1 cm quartz cuvette and stirred at 120 rpm to generate a heterogeneous velocity gradient (heterogeneous flow). The shearing protocols applied are presented in table 5.1. Fibril formation was probed via ThT fluorescence enhancement, using excitation and emission wavelengths of 450 and 482 nm respectively, and deemed complete when the fluorescence intensity had attained a stable plateau value.

Table 5.1: Description of the different shearing protocols.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Experimental Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Sheared at 37°C for ~3 h. Stopped shearing when I &lt; I_0. Incubated at 37°C for a further ~24 h.</td>
</tr>
<tr>
<td>b</td>
<td>Sheared at 37°C for ~4 h. Stopped shearing when I = I_0. Incubated at 37°C for a further 24 h.</td>
</tr>
<tr>
<td>c</td>
<td>Incubated at 37°C for 27 h without shearing. Sheared at 37°C for a further 21 h.</td>
</tr>
</tbody>
</table>

Circular dichroism: The secondary structural content of Aβ_{40} reaction solutions, at defined time-points during incubation, was studied using far-UV CD measurements. Data were collected using the J-815 spectropolarimeter at room temperature. Solvent spectrum
(i.e. phosphate buffer and ThT) was used as baseline for data collection. Each spectrum was an average of three scans.

Atomic force microscopy: The morphology of species in the reaction samples were studied using the MFP 3D AFM set in tapping mode. Aliquots (20 µL) of the reaction samples were applied to freshly cleaved mica and allowed to dry in a laminar flow cabinet. Excess Aβ₄₀ solutions were rinsed off using Milli-Q water. The mica was then dried using nitrogen gas before taking images of the samples.

5.3 Results

![Graph of Aβ₄₀ fibril assembly kinetics](image)

Figure 5.1: The effect of stirring on the kinetics of Aβ₄₀ fibril assembly. Protocols a to c are described in table 5.1. Sheared and unsheared controls are also shown. The time-scale for protocol c was normalised to correspond to the time when shearing was started.

The effect of heterogeneous shear flow on the kinetics of Aβ₄₀ aggregation was studied by performing a series of experiments where shearing was stopped, or started, at discrete time-points during the three-stage kinetic process of Aβ₄₀ fibril assembly. The protocols described in table 5.1 were used. The aim was to determine the effect of shear at each stage of Aβ₄₀ fibrillogenesis.
**Aβ₄₀ fibrillation in heterogeneous flow**

Fluorescence profiles of the various Aβ₄₀ reaction solutions are shown in figure 5.1. The kinetic profile of a continuously stirred sample showed a sigmoid curve, which is characteristic of a nucleation-dependent fibril assembly (47). The lag phase is the time taken for the evolution of fibril-competent nuclei. The steep slope translates to the rapid assembly of fibril nuclei into multimeric fibrillar aggregates. This is followed by a plateau, symptomatic of an equilibrium phase. The observed kinetic trend is consistent with similar studies reported for an array of polypeptides (161). AFM height trace images of the reaction sample show a morphological transition of an initially monomeric Aβ₄₀ (height: ~1.5 nm) to a mature fibril structure (height: ~6 to 10 nm) (figure 5.2). Secondary structurally analysis, using CD spectroscopy, confirmed the initial random coil solution-conformation of Aβ₄₀, and the enriched β-sheet content of its fibrillar aggregates (32). In the absence of agitation, fibrils did not form over the same time frame as the continuously stirred sample (figure 5.1), and even after three days of incubation at 37°C. However, others have shown that quiescent oligomeric aggregates eventually form fibrils over an extended period of time (228).

Figure 5.2: AFM height trace images of Aβ₄₀. Aliquots of the sheared control sample were imaged: (a) before the start of the experiment; and (b) after 24 h of continuous stirring at 37°C. Scale bars represent 1 μm.

It is noteworthy that on shearing, the emission intensity of ThT decreased for the duration of the lag phase of Aβ₄₀ aggregation (i.e. I < I₀), during which globule-like aggregates develop. This observation is attributed to the temperature dependence of ThT fluorescence (229). That is, by increasing the temperature to 37°C, the emission intensity
of ThT diminishes. However, as fibrillar aggregates evolve, the ThT molecules interact with the multimeric β-sheets, leading to a rapid enhancement of the emission intensity at 482 nm (43).

**Heat-induced fibril formation**

In protocol a, the Aβ_{40} solution was sheared at the start of the experiment, and shearing was ceased partway through the lag phase (~3 h; I < I_0), when the solution contained mainly fibril-competent oligomeric aggregates with an average height of 3 nm (figure 5.3a1). This inference was drawn based on the emergence of protofibrils in the sample. The aim was to investigate whether stirring was necessary to convert these aggregates into mature fibrils.

Upon cessation of shearing, the ThT fluorescence emission of the reaction solution demonstrated a slow but consistent increase in intensity over the experimental time frame of 27 h (figure 5.1), which indicates a sluggish rate of fibril assembly. Interestingly, the kinetic profile did not show the characteristic sigmoid curve of a nucleation-dependent growth. An AFM image of the sample at 27 h showed mostly protofibrils, with oligomeric aggregates still visible in the background (figure 5.3a2). The protofibrils had a beaded morphology(29) and an average height of ~3.0 nm, similar to that of the globule-like oligomeric aggregates.

Clearly, the development of the protofibrils was at the expense of the globule-like aggregates. In effect, the rate of fibril growth was much slower than when the sample was sheared continuously, but faster than when the sample was not sheared at all. Furthermore, it was interesting to note that once fibril-competent nuclei had evolved, thermal treatment alone facilitated their morphological transition into protofibrils.
Moore et al. (227) had previously reported that roughly spherical peptide assemblies (oligomeric aggregates) interact directly with the growing fibril. Harper et al. (230) suggests that protofibril growth may involve the coalescence of smaller aggregates. The height match of the oligomeric aggregates to protofibrils, together with the observed inverse relationship between the number of protofibrils and oligomeric aggregates absorbed on mica, support the general consensus that the aggregates serve as the building blocks for fibril assembly (29, 47, 153, 226, 230).

The CD spectrum on cessation of shearing shows a spectral minimum at 197 nm, indicative of a predominantly random coil structure, while the reaction sample at 27 h
shows a broad minimum between 208 and 220 nm, which suggests a mix of α-helix and β-sheet conformations (figure 5.4, protocol a).

In protocol b, the amyloidogenic solution was sheared for ~4 h, at which time the ThT emission intensity had began to rise (i.e. $I/I_0 = 1$). The sample at this point contained a significant proportion of protofibrils (figure 5.3b1) and the initial random coil structure was now dominated by an α-helix conformation (figure 5.4; protocol b). Interestingly, the ThT emission intensity continued to rise after cessation of shearing, showing the characteristic sigmoid curve (figure 5.1). However, the assembly phase progressed at a much slower pace relative to when the sample was sheared continuously, but much faster compared to when the solution contained mainly aggregates (protocol a, $I/I_0 < 1$).

After the ThT fluorescence had reached a plateau, the solution contained predominantly mature fibrils (figure 5.3b2), and rich in the characteristic β-sheet structure observed in amyloid fibrils (figure 5.4; protocol b). These observations demonstrate that once a critical number of protofibrils develop in solution, polymerization into fibrillar aggregates proceeds even in the absence of shearing, albeit, at a relatively slow pace.

**Stirring accelerates the conversion of globule-like aggregates to fibrils**

In protocol c, the amyloidogenic solution was thermally treated without shearing for 27 h. Similar to the unsheared control, the sample contained only roughly spherical aggregates (figure 5.3c1), ~2.5 nm high, with no protofibrils on the mica surface. These aggregates showed a predominantly random coil conformation (figure 5.4, protocol c) similar to monomeric $\text{A}^\beta_{40}$. The aim here was to ascertain whether these oligomeric aggregates serve as the subunits for fibril assembly, and whether stirring facilitates their conversion into mature fibrils. The pre-heated solution was thus sheared for a period of 24 h.

Shearing the globule-like aggregates triggered an immediate and rapid increase in ThT emission intensity, with no detectable lag phase (figure 5.1), reaching a plateau within a relatively short time frame. This suggests instantaneous fibril assembly using the oligomeric aggregates as starting material. The solution at the equilibrium phase
contained mature fibrils (figure 5.3c2) rich in β-sheet (figure 5.4; protocol c). Clearly, Aβ_{40} aggregates serve as the nucleus for fibril assembly, and the rate of assembly is facilitated by shearing.

![Figure 5.4: CD spectra of Aβ_{40} solutions exposed to the different shearing protocols. The spectra correspond to the protocols and images described in table 5.1 and figure 5.3 respectively.](image)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Wavelength (nm)</th>
<th>Pre-heated (random coil)</th>
<th>Pre-heated then stirred (β-sheet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>190 195 200 205 210 215 220 225 230 235 240</td>
<td>I/I₀ &lt; 1 (random coil)</td>
<td>27 h α-helix + β-sheet</td>
</tr>
<tr>
<td>b</td>
<td>0 h (random coil)</td>
<td>I/I₀ = 1 (α-helix)</td>
<td>28 h (β-sheet)</td>
</tr>
<tr>
<td>c</td>
<td>0 h (random coil)</td>
<td>I/I₀ = 1 (α-helix)</td>
<td>28 h (β-sheet)</td>
</tr>
</tbody>
</table>

Figure 5.4: CD spectra of Aβ_{40} solutions exposed to the different shearing protocols. The spectra correspond to the protocols and images described in table 5.1 and figure 5.3 respectively.
5.4 Discussion

Senile plaques composed predominantly of amyloid-β aggregates are a pathological hallmark of Alzheimer’s disease (153). Research into the aetiology of the disease has targeted the mechanism of fibril formation, with emphasis on the toxicity of intermediate species (34). In addition, environmental factors that enhance amyloid fibril formation are considered to be essential (231). That is, conditions that destabilize the native conformation of a peptide/polypeptide chain are believed to be critical in protein aggregation.

**Fibril formation in heterogeneous flow**

The results presented in this chapter show that the kinetics of Aβ40 aggregation are greatly enhanced when the peptide is exposed to heterogeneous flow. This mode of agitation had a profound effect on both the nucleation phase (which involves the formation of oligomeric aggregates) and the rate of fibril assembly. The possible mechanisms involved in this process are discussed below.

The non-uniform flow velocity of Aβ40 solutions resulting from stirring, and the associated fluctuating stresses, trigger the mechanical destabilization of native Aβ40, enhancing the nucleation of monomers into oligomeric aggregates and their subsequent rapid polymerisation into prefibrillar aggregates (protofibrils). The results (figure 5.3c1 and 5.3c2) clearly show that the rapid fibril development is facilitated by orthokinetic aggregation on stirring. In fact the observed slow rate of fibril assembly from oligomeric aggregates in quiescent solutions corroborates this conclusion. It is interesting to note that the formation of protofibrils exacts a structural reorganization of the polymerizing aggregates. This feature is characterized by the folding of segments of an initially random coil structure into an α-helix conformation.

The mixing effect of stirring promotes the continuous polymerization of fibril-competent nuclei as well as the cross-linking of protofibrils, giving rise to mature fibrils. The morphological transformation from protofibrils to mature fibrils also features a conformational transition from α-helix to a predominantly β-sheet conformation,
ubiquitous in amyloid fibrils. The hydrodynamic stress produced by stirring is known to promote ordering of chain elements in crystallization nucleation within a melt (232). The stress in the flow field may also trigger the fragmentation of mature fibrils, providing additional active ends which serve as ‘seeds’ for fibril assembly. It has previously been reported that rotation accelerates both the lag and assembly phases of fibril formation in amyloidogenic Sup35 solutions (47).

Others have shown that agitation enhances the air-liquid interface in solutions (111), and it is well established that peptides/polypeptides show structural susceptibility to interfacial forces at such polar/nonpolar boundaries (233). Several tiny gas bubbles were observed on the stirrer bar each time stirring was ceased. It is possible, therefore, that by a mechanism similar to cavitation, the magnetic stirrer bar generates millions of micro-gas-bubbles that migrate towards the fluid surface, hence increasing the gas-liquid interface throughout the peptide solution. Sluzky et al. (105) reported the important role of hydrophobic interfaces, in particular air-water and teflon-water interfaces, in insulin aggregation. The amphiphilic nature of Aβ₄₀ predicts that monomers will accumulate at the air-water interface, resulting in structural destabilization; potentially leading to aggregation (15). This is consistent with recent reports that Aβ aggregation is greatly enhanced at a polar/non-polar interface compared to Aβ in bulk solution (224).

Taken together, the combination of a heterogeneous flow profile and an increase in air-water interface, resulting from stirring, promotes the destabilization and subsequent aggregation of monomeric Aβ₄₀ into amyloid fibrils. In addition, thermal treatment of the solution reinforces the hydrophobic driving force promoting aggregation (234).

Interestingly, when Aβ₄₀ solutions are exposed to uniform shear flow, as opposed to heterogeneous flow, the end-point of the aggregation process is the formation of protofibrils (77). That is, the development of mature fibrils is not observed. A similar observation was made on comparing β-lactoglobulin aggregates generated in heterogeneous and uniform flow fields (73). It is suggested that the hydrodynamic drag in uniform flow fields limits the frequency of association of fibril-competent nuclei, and
hence their polymerization into mature fibrils (73). However, as previously stated, the mixing nature of heterogeneous flow facilitates orthokinetic aggregation of fibril-competent nuclei into mature fibrils.

**Thermal contributions to Aβ_{40} aggregation**

The results show that heating alone induced the formation of oligomeric aggregates, but did not trigger fibril formation even after 72 h of incubation. However, thermal treatment of shear-enhanced fibril-competent nuclei resulted in the formation of protofibrils (figures 5.3a1 and 5.3a2), and the conversion of protofibrils to mature fibrils (figures 5.3b1 and 5.3b2). Heat-triggered conversion of protofibrils to mature amyloid fibrils has been reported for β_{2}-microglobulin (235), and Aβ_{40} protofibril elongation is known to be temperature dependent (230). Heating causes increased β-sheet content and gelation of bovine serum albumin solutions(111), and has been shown to convert preformed aggregates of β_{2}-microglobulin and hen egg-white lysozyme into mature fibrils (228). In this work, it was observed that Aβ_{40} solutions show rapid aggregation when incubated at 37°C compared to incubation at 25°C. It has been reported that heat-induced partial denaturation of proteins is occasionally accompanied by an irreversible process that results in aggregation (235). This may well explain the observed temperature-induced Aβ_{40} aggregation under quiescent conditions.

Oligomeric Aβ aggregates have been found in the cerebrospinal fluid of AD patients compared to age match controls (236). Hence, although amyloid assembly *in vivo* may be mediated by several factors (36), preformed aggregates could develop into mature fibrils latently under physiological conditions (228). Taken together, a combination of heterogeneous shear flow and thermal treatment provide complementary conditions that not only destabilize native Aβ_{40}, thereby increasing its propensity to fibrillate, but also facilitate the aggregation process.

**Intermediates in Aβ_{40} fibril assembly**

The morphology and secondary structural content of Aβ_{40} solutions, during the time-course of aggregation, were studied using AFM and CD respectively. The results show
that the path to amyloid fibril formation involves a sequence of conformational and morphological changes. The observed intermediate species that preceded fibril formation were oligomeric aggregates, which maintained the random coil structure of the native peptide, and protofibrils, which contained a balance of α-helix and β-sheet structure. It is proposed that the oligomeric aggregates are large nucleation units composed of between 5-12 monomers (29, 237) with an average height of ~3.0 nm. Studies suggest that these aggregates form via classic colloidal coagulation (50), and that their disordered state is attributable to the non-specific hydrophobic interactions that drive their coalescence (238). From figure 5.3, it was evident that the oligomeric aggregates, which formed rapidly in the presence or absence of agitation, evolved into the more structured protofibrils, thought to contain about 1500 Aβ\textsubscript{40} monomers (224).

The mechanism of protofibril formation involves the directional coalescence of oligomeric aggregates, via hydrophobic interactions, and a structural reorganization (driven by the formation of directional inter-peptide hydrogen bonds) (238) from a disordered state to a more defined secondary structure (α-helix/β-sheet) (230). AFM analysis of Sup35 aggregation suggest that fibrils elongate by a mechanism in which spherical oligomers dock on the growing fibril ends, followed by a conformational conversion to consolidate the amyloid structure (239). Indeed, proteins have directionality conferred on them by virtue of the N- and C-terminals of the main chain.

The continuous polymerization and cross-linking between protofibrils gives rise to mature fibrils (29). The absence of oligomeric aggregates at the end-point of fibril formation (figures 5.3b2 and 5.3c2) supports the fact that these aggregates are involved in the morphological and structural transitions that culminates in the formation of the fibril structure. Xu et al. (240) reported that fibril formation is a result of the association of spherical oligomeric aggregates, containing 8- to 10-monomers. Taken together, fibril assembly in Aβ\textsubscript{40} occurs primarily, if not entirely, by the association and conformational conversion of oligomeric aggregates at the growing fibril ends.
Rate-determining morphological species in Aβ₄₀ fibril assembly

One of the critical issues in kinetic studies of amyloid fibril assembly is to establish the rate-limiting step. This typically involves the formation of a fibril nucleus beyond which a spontaneous feed-forward reaction results.

A number of different rate-limiting steps have been proposed for fibril assembly. Serio et al. (47) suggests that the rate-limiting step in amyloid assembly is the evolution of a fibril-competent nucleus (oligomeric aggregates), excluding conformational conversion. However, it is argued that the strong dependence of lag time on protein concentration makes the acquisition of a β-sheet structure the rate-limiting step in fibril assembly (241).

Considering the results presented, it seems that in Aβ₄₀ fibrillation, the rate-limiting morphological species depends on whether the sample is sheared or otherwise. In agitated Aβ₄₀ solutions, the formation of fibril-competent oligomeric aggregates is the rate-limiting step in the fibril forming process. Once these aggregates are formed, fibril assembly proceeds rapidly. In the absence of agitation, however, the formation of a critical number of protofibrils, in conjunction with the acquisition of a β-sheet structure, consolidates fibril assembly in quiescent Aβ₄₀ solutions. Indeed, it has been reported that agitation alters the rate determining step of amyloid assembly, which restricts comparing the kinetics of agitated to non-agitated amyloidogenic solutions (226).

Mechanism of fibril formation in heat-treated Aβ₄₀ solutions in heterogeneous flow

Based on the results presented in this study, a schematic model of Aβ₄₀ fibril assembly under thermal and/or agitated conditions is proposed (figure 5.5). The fibrillogenesis of Aβ₄₀ proceeds via three principal stages: (i) structural perturbation and consequent aggregation of monomers to form a fibril-competent nuclei; (ii) linear association of these nuclei to form ‘beaded’ protofibrils; and (iii) fusion and structural reorganisation of protofibrils into mature fibrils. Shearing accelerates the first two stages, but inhibits the third.
The rate of Aβ40 aggregation is enhanced on exposure to heterogeneous flow, and this is augmented by raising the solution temperature to 37°C. Both heat and shear treatment may trigger structural destabilization of the solution conformation of Aβ40 via thermal and mechanical perturbation respectively. The perturbed states may expose bonding sites that facilitate intermolecular interactions between monomers, leading to aggregation (242). The aggregates grow in size until they are fibril competent, composed of between
5- and 8-mers, a size consistent with a β-crystallite (29, 243), and serve as the nucleating centres for fibril assembly.

5.5 Conclusion
Amyloid fibril formation in Aβ40 was consistent with two of the mechanistic models proposed for fibril assembly; the nucleated-polymerisation model proposed by Jarrett et al. (46) and the nucleation-conformational-conversion model proposed by Serio et al. (47). Aβ40 fibril assembly was enhanced on exposure to heterogeneous flow relative to quiescent samples. It was established that the aggregates serve as the building blocks in Aβ40 fibril assembly, and their formation is the rate-determining step in agitated Aβ40 solutions. Flow-induced Aβ40 aggregates formed mature fibrils latently, and this may have some physiological relevance as Aβ aggregates have been found in the cerebrospinal fluid of AD patients (230).
6. Shear-induced Unfolding of Bovine Insulin in Couette Flow

6.1 Introduction

Mechanical destabilization of the higher order structure of a native protein often leads it to misfold and coalesce into insoluble aggregates (14). Agitated protein solutions have been shown to aggregate more quickly relative to quiescent samples (15, 111, 225). A number of studies consider hydrodynamic shear stress as the trigger of protein deformation and subsequent aggregation in agitated protein solutions (9, 54, 102, 104, 112, 233). However, it remains to be known whether shear stress directly disrupts native protein molecules, hence, initiating aggregation (112) or its influence stems from the continuous generation of an air-liquid interface, where interfacial tension forces trigger protein unfolding and subsequent aggregation (233). Whatever the case may be, the mechanical effect is purported to proceed via conformational disorder in the native protein, resulting in its deformation and subsequent exposure of hydrophobic regions to the aqueous environment (104). Deformed protein molecules associate primarily via hydrophobic interactions, and precipitate out of solution as amorphous and/or fibrillar aggregates (15, 159). This observation is a major concern especially in the bioprocessing of protein therapeutics, such as insulin and immunoglobulin G, because their exposure to shear during pumping, centrifugation, filtration, and fractionation makes them aggregation prone (158).

Intriguingly, accumulating evidence associates the deterioration of vascular microcirculation with the onset of protein conformational disorders such as Alzheimer’s disease (64, 65). The pathological hallmark of this, and related conformational disorders, is the precipitation of proteinaceous elements into well structured, insoluble fibrillar aggregates. Alterations in haemodynamic shear stress on arterial walls participate in the pathogenesis of atherosclerosis, which involves the deposition of proteinaceous material in the form of artheromatous plaque (13, 66). Shear-dependent unfolding of protein components in the three-dimensional structure of human von Willebrand factor has also been reported (244). Furthermore, in vitro studies on well defined food and drug protein systems, including β-lactoglobulin and insulin, show that these proteins form large
aggregates/fibrils under shear (54, 104, 113). It is interesting to note that fibrillar insulin aggregates have been reported both in patients with type II diabetes, especially after repeated administration of the protein-drug (170), and in normal aging (171).

Although there has been progress in investigating the influence of shear flow on protein aggregation, little work has been expended on the effect of shear on a protein’s secondary or tertiary structure (104) as determined by fluorescence and circular dichroism spectroscopy. Hence, current literature fails to address the relationship between shear and the extent of deformation of the higher order structure of a native protein; a prerequisite for aggregation (14, 102). In the preceding chapter, it was observed that the rate of Aβ aggregation is enhanced in heterogeneous flow. It was concluded that the stress generated in the flow field accelerated the nucleation and polymerization of amyloidogenic Aβ oligomers into protofibrils, but inhibited their fusion into mature fibrils. Here, a well defined flow field (Couette flow) is employed to characterize the conformational dynamics of insulin, a natively folded protein, under hydrodynamic stress in simple shear flow. This is an improvement over the non-uniform shear regimes in the flow field employed in the preceding chapter, and by others (15, 104), in investigating the shear-stability of peptide/polypeptide systems. The advantage here is that the shear rate, hence hydrodynamic stress, acting on insulin molecules in solution is uniform in time and space. More importantly, the structural changes in native insulin are monitored in situ and in real time using intrinsic Tyr fluorescence and CD spectroscopy respectively. This allows both qualitative and quantitative structural analysis of the effect of shear stress on a natively folded protein. Indeed, understanding the rheological properties of proteins is of critical importance in defining their conformational and structural attributes in flow.

Bovine insulin was chosen as the model protein for this study because: (i) it is a well characterized protein system; (ii) it is economical and easy to use; and (iii) it has been shown to form aggregates under shear.
6.2 Materials and methods

Bovine insulin was procured from Sigma (St. Louis, MO) and used without further purification. All other chemicals were of analytical grade.

*Sample preparation:* Bovine insulin was dissolved directly in 0.1% HCl (pH 1.9) to yield a concentration of ~0.2 mg/mL. The final insulin concentration was calculated from UV absorption at 280 nm, applying a molar extinction coefficient of 5.53 mM⁻¹ cm⁻¹.

*Couette cell:* Both fluorescence and CD experiments were performed using the Couette cell described in chapter 3. Insulin solutions were exposed to shear rates of 200, 400 and 600 s⁻¹, which are within physiological range. Arterial wall shear rates are estimated to range between 134.2 and 1640 s⁻¹ (223). The studies were conducted in a temperature controlled room (~20°C) to limit thermal contributions to the results.

*Fluorescence studies:* The Varian Cary Eclipse fluorometer was set to kinetic mode for the fluorescence experiments. The excitation and emission wavelengths were set at 276 and 305 nm respectively, to monitor the intrinsic Tyr fluorescence of the insulin sample. This idea was borrowed from chapter 4, where the intrinsic fluorescence of insulin was found to be sensitive to structural changes in the bulk solution. An attempt to apply the amyloid dye, ThT, to monitor insulin aggregation did not yield good results, since no significant fluorescence enhancement was observed over the duration of the experiments. This is possibly because very few fibrillar aggregates, and most often none, developed in the sheared solutions.

*Circular dichroism and SDS-PAGE studies:* CD data were acquired using the CD instrument previously described (Chapter 3). The instrument was set to the interval scan mode for data collection. A data interval of 0.1 nm, a bandwidth of 1.0 nm, response time of 4 s, and a scanning speed of 100 nm/min were used. Samples recovered from CD experiments were analysed for fragmentation using SDS-PAGE.
**Atomic force microscopy:** The morphology of sheared insulin samples were analysed using the asylum MFP 3D AFM. Images were acquired using tapping mode in air. The sample preparation was the same as that described in the preceding chapters.

### 6.3 Results

![Figure 6.1](image_url)

**Figure 6.1:** Shear-effect on Tyr fluorescence emission intensity of bovine insulin in flow. The emission intensity of Tyr molecules was measured for insulin samples (0.2 mg/mL) sheared at 200 s⁻¹ (▲), 400 s⁻¹ (◆), and 600 s⁻¹ (■) over 2 h. The figure shows a time dependent decrease in Tyr fluorescence intensity in response to flow. The flow effect is more pronounced at high shear rates.

Figure 6.1 shows shear-dependent changes in the intrinsic Tyr fluorescence of bovine insulin in flow. For each profile, an initial sharp decrease in Tyr fluorescence was observed at the onset of shear. This effect was more pronounced with increasing shear rate. At shear rates of 200 s⁻¹ and 400 s⁻¹, the intrinsic fluorescence decay approaches equilibrium after ~30 min, whereas at 600 s⁻¹, it shows complete saturation after ~10 min. The observed fluorescence decay indicates changes in the microenvironment of Tyr residues, resulting from destabilization of the entire protein structure (39).
four Tyr residues in bovine insulin are located in helical segments contained in hydrophobic pockets of the protein(159), and the emission intensity at 305 nm is an average of the parametric changes affecting the microenvironment of all Tyr residues. Therefore, the observed fluorescence decay is explained by a shear-induced disruption of the helical segments of bovine insulin, and subsequent exposure of hitherto sequestered Tyr residues to the aqueous environment. The extent of fluorescence decay reflects the degree of structural disruption. Qualitatively, the data shows a relationship between the magnitude of shear rate applied and the resultant structural deformation of bovine insulin.

For a quantitative assessment of the structural perturbation of insulin molecules in shear flow, the far-UV CD spectrum of insulin solutions was monitored in real time under Couette flow. Figure 6.2 shows time-dependent CD spectra of insulin solutions exposed to different shear rates. At ambient temperature, the far-UV spectrum of native insulin showed two negative peak intensities at 209 nm and 222 nm, typical of its dominant α-helical solution conformation. At the onset of a given shear rate, this spectrum diminished in intensity as a function of time, indicating the unfolding of helical segments of native insulin molecules. The observed structural deformation was more drastic with increasing shear rate. For example, at 200 s$^{-1}$ (figure 6.2, panel A), insulin retained its helical structure even after 40 min of shear exposure, although weakened in intensity, whereas at 400 s$^{-1}$ and 600 s$^{-1}$ (figure 6.2, panels B and C), the helical content had diminished considerably within 15 and 8 min of shear exposure respectively.
Figure 6.2: CD spectra showing insulin deformation in flow. The figure shows insulin samples treated at shear rates of: (A) 200 s$^{-1}$ at 0, 3, 8, 10, 13, 15, 20, 28, 33, and 38 min; (B) 400 s$^{-1}$ at 0, 3, 5, 10, 15, 25 and 33 min; and (C) 600 s$^{-1}$ at 0, 5, 8, 20, 30 min. The black arrows on each panel show both the direction of increasing time and decreasing negative ellipticity at $\theta_{222nm}$. 
Figure 6.3: Shear-dependent changes in the helicity of bovine insulin in flow. Insulin samples were sheared at 200 s$^{-1}$ (■), 400 s$^{-1}$ (●) and 600 s$^{-1}$ (▲). The figure shows the percentage of initial α-helix remaining (change at 222 nm) as a function of time. A shear rate-dependent unfolding of helical segments of bovine insulin is observed, with a more pronounced effect occurring at high shear rates.

The CD data was further analysed by plotting the shear-dependent helicity change of bovine insulin as a function of time (figure 6.3). An initial helix content of 56% was estimated for bovine insulin, similar to that of its human homologue. This was not surprising since the sequence differences between the two are not thought to affect structural aspects or ligand binding properties of wild-type insulin (245). The helicity of 56% indicates that ~29 of the 51 amino acid residues makeup the helical moieties of bovine insulin (i.e. 51 total residues x 0.56). The initial 30 min of shear exposure was considered because much of insulin deformation occurred within this time frame. At 200 s$^{-1}$, the helicity showed a gradual decrease from 56% down to 26% in the initial 30 min of shear. At 400 s$^{-1}$ and 600 s$^{-1}$, half the helicity (56% to 28%) was lost after 9 min and 6 min respectively. The observed decrease in helicity was obviously more drastic at 400 s$^{-1}$ and 600 s$^{-1}$, with minima of 16.7% and 6.6% respectively, at 30 min of shear exposure. Therefore, out of the initial 29 helical moieties, the number remaining after the first 30 min of shear exposure was approximately 14, 8 and 3 in order of increasing shear rate.
The absence of a lag time was obvious in the three profiles, which clearly shows that the structural deformation of insulin occurred at the onset of shear. It is noteworthy that the CD data substantiate the conclusions drawn from the fluorescence experiments in figure 6.1.

![Figure 6.4: SDS-PAGE analysis of pre- and post-shear insulin samples.](image)

To investigate whether the insulin samples fragment under the shear rates applied, SDS-PAGE analysis of pre- and post-shear insulin solutions were performed after CD measurements (figure 6.4). Both samples gave a similar band, which provides qualitative evidence that the primary structure of insulin monomers was maintained during shear exposure.

Figure 6.5 shows AFM analysis of post-shear insulin samples obtained from figure 6.1. The insulin sample sheared at 200 s$^{-1}$ contained aggregates ranging from 3 nm to 50 nm in height. Similar aggregates were observed in the sample treated at 400 s$^{-1}$, but were
sparsely distributed. Interestingly, the sample exposed to a shear rate of 600 s\(^{-1}\) contained relatively small aggregates and fibrillar forms.

![Figure 6.5: AFM height trace images of insulin aggregates formed in shear flow. Images were obtained from insulin samples sheared for 2 h at (A) 200 s\(^{-1}\), (B) 400 s\(^{-1}\) and (C) 600 s\(^{-1}\). The black arrows in panel C show fibrillar insulin aggregates. Scale bars represent 1 \(\mu\)m.](image)

**6.4 Discussion**

Like other polymer solutions, aqueous bovine insulin exposed to simple shear flow experiences a blend of rotational and deformation velocities of equal intensity in the direction of flow (58). In the rotational flow field, insulin molecules go through whole body rotation, align in the direction of flow, and experience zero shear strain, hence, maintain their native conformation. The alignment of molecules in uniform shear stress fields has previously been observed in human von Willebrand Factor(244), and a single DNA chain (58). However, when insulin molecules cross the local plane of zero shear, the hydrodynamic drag in the deformation flow field trigger structural destabilization (9). For simplicity, the deformation cycle in simple shear flow is decomposed into extensional and compressive (tumbling) events of equal magnitude. It is expected that the helical segments of native insulin will unfold in the extensional flow component of the deformation cycle. This occurs when the hydrodynamic drag in the deformation flow field overcome the intramolecular hydrogen bonds stabilizing the helical structure of
native insulin. This event exposes hydrophobic regions in native insulin to the aqueous environment.

As individual insulin molecules tumble end-over-end, due to rotational diffusion, in the flow field, the pressure from compression facilitates the rapid association of deformed insulin molecules, via hydrophobic and electrostatic interactions, into oligomeric aggregates (113). It was observed that the aggregate size diminished with increasing shear rate, which shows that in high shear regimes, the stress generated in the deformation cycle (specifically the extensional flow field) breaks-up aggregates that are loosely associated. This is not surprising because the initial bonds formed between deformed protein molecules are known to be weak(246), and an increase in shear rate boosts the frequency of stretching events in the flow field (247). In addition, the flow velocity increases with increasing shear rate. Hence, a consequent increase in the hydrodynamic shear stress acting on insulin monomers/aggregates is expected.

The development of fibrillar aggregates at 600 s\(^{-1}\) is explained by increased polymerisation of deformed bovine insulin monomers in the flow field. This is consistent with the observation that shear promotes ordering of chain elements in crystallization nucleation within a melt (232). Indeed, empirical modelling of sheared protein solutions attribute conformational changes and accelerated aggregation to high shear rates (248). The stability of these aggregates stems from the extensive network of hydrogen bonds between \(\beta\)-strands found in fibrillar forms. It is interesting to note that the fibrillar insulin aggregates reported here are much shorter and are sparsely distributed compared to those formed in heterogeneous flow at elevated temperature (chapter 4). This suggests that both temperature and flow conditions influence insulin fibril assembly, similar to the case of amyloid-\(\beta\) in the preceding chapter and other protein systems reported by others (113, 249).

Taking into account the data presented in the preceding chapter and that reported elsewhere(114), the paradoxical effect of simple shear flow on protein aggregation is obvious. That is, it triggers protein deformation, which is critical to fibril formation, but
also inhibits the development of mature fibrils due to the rotational component of the flow field, which interrupts the formation of polymeric fibrillar aggregates.

The Couette shear exposure used in the current study is less complex than that experienced by insulin in pulsatile blood flow. Blood plasma contains both cells and a range of proteins, and is generally modelled as a Bingham plastic with Newtonian viscosity in flow. A high level of confinement is observed under physiological flow in capillaries and in the filtering organs (223). The shear rates experienced \textit{in vivo} will therefore vary over a wide range and be complex (223). For example, haemodynamic shear rates in partly clogged arterioles approach $10^4 \text{ s}^{-1}$ (250). In addition, others have shown that molecular confinement accelerates the deformation of entangled polymers under squeeze flow (251); a condition comparable to blood flow. The calculated Reynolds number for individual bovine insulin molecules is $R_e \sim 4 \times 10^{-3}$, and $R_e \sim 1$ for the bulk solution at the highest shear rate applied. This assumes that blood has a Newtonian viscosity of 0.1 Pa.s$^{-1}$ and density of water. Since these values are relatively small, it is concluded that the flow conditions applied in this study were laminar in nature. In fact, laminar flow patterns have been observed at shear rates as high as 1300 s$^{-1}$ using this Couette cell (57).

\textbf{6.5 Conclusion}

A novel real time analysis of the effect of shear flow on the structure and conformation of bovine insulin in a well defined flow field was performed. The results show that the protein experiences structural destabilization (decrease in helicity) upon shear-exposure, leading to intra-/intermolecular complexation (aggregation) arising from hydrophobic interactions. This effect was observed at the onset of shear and was marked at higher shear rates. Large insulin aggregates were observed in low shear regimes with fibrillar forms evolving in high shear regimes. Hence, the deformation velocity in fast flow fields retards the formation of large aggregates, but promotes the ordering of deformed insulin molecules into the more stable fibrillar forms. The present information might help explain the mechanism of shear-induced protein deformation and consequent plaque deposition \textit{in vivo}. 
7. $\alpha$-Helix Unfolding in Simple Shear Flow

7.1 Introduction
Astbury was the first to show molecular level conformational changes in proteins due to applied stress (252). Elastic stretching of keratin fibres in combination with X-ray scattering led Astbury and Woods to determine that a helix to β-sheet transition occurred under large strain (252). This insight led Pauling and Corey to later determine the helical structure accurately using X-ray scattering (253, 254). Since the seminal study by Astbury and co-workers, a large body of work has been directed to understanding the effect of stresses on synthetic and biological molecules. The work of Smith et al. and LeDuc et al. has been instrumental in developing our understanding of the effect of hydrodynamic forces on DNA molecules, and polymers as a whole (58, 255).

A number of recent publications have shown that hydrodynamic forces do indeed induce conformational changes in proteins and cause unfolding events to occur (9, 68, 75, 100). In the preceding chapter, the helical segments of bovine insulin were observed to unfold in simple shear flow, leading to intermolecular complexation (aggregation). Given that protein conformation is critical in their function, these findings have significant implications for much of biology. Furthermore, the effects of agitation and shear flow have also been known to accelerate amyloid fibril formation (15, 47, 54, 112, 113).

Several recent studies have shown the presence of $\alpha$–helical intermediates in the pathway to fibril formation by several proteins (256-259). However, the role of the $\alpha$-helical intermediate in amyloid formation is not understood. Given that $\alpha$–helices are a very important and common secondary structural feature of a significant number of proteins(260), understanding their role in protein unfolding and fibril formation would seem a worthwhile endeavour. Here, the unfolding of helical segments in simple shear flow is further analysed using the model system, poly-L-lysine (PLL), which forms predominantly $\alpha$-helical structures at high pH (120, 174). A range of molecular weights have been investigated by exposure to defined Couette flow while measuring the circular dichroism spectra in real time. Critically, the results show that the $\alpha$–helices unfold in
flow where the strain is the key parameter in determining the unfolding. The specific nature of the protein tertiary structure will be instrumental in stabilising the helices against flow induced unfolding. The findings have significant implications for proteins in flow. It is noteworthy that the commercial availability of various molecular weights of PLL, needless to say, of the same chemical composition, affords a direct comparison of the chain-length dependence of the polypeptide in response to perturbation. For example, thermal studies on selected molecular weights of PLL show that the helix-to-sheet transition temperature decreases with increasing chain-length (149).

7.2 Materials and Methods

Materials: Four molecular weights of poly-L-lysine hydrobromide were studied. The samples were purchased from Sigma-Aldrich (St. Louise, MO) with a supplier reported molecular weight average (by viscosity) of 15, 68.3, 205.7 and 381.2 kDa. The polypeptides were prepared by dissolution in a triple-distilled water/NaOH mixture (pH 11.7), pre-cooled and filtered (0.22 µm filter), to give a final working concentration of ~0.2 mg/ml. The solution concentration of the PLL samples was calculated using the UV absorption at 214 nm and methods described by Kuipers and Gruppen (261). The samples were stored at 4°C, but allowed to equilibrate to room temperature (~20°C) before use.

Couette flow cell: Shear experiments were performed in the custom built quartz-flow-cell of Couette geometry previously described (chapter 3), with a gap size of 0.021 cm (54, 59). PLL samples were exposed to shear rates of 74, 117, 219, 302, 518 and 715 s⁻¹ for 1 h. At these shear rates, laminar flow patterns are achieved in the quartz-flow-cell. The flow cell was mounted and aligned in the J-815 CD spectropolarimeter (Jasco, Tokyo, Japan) such that CD data were recorded in real-time during shearing, similar to that of bovine insulin described in the preceding chapter.

CD measurements: Far-UV CD measurements were performed over the wavelength range of 190 to 240 nm in a temperature controlled room (~20°C) to limit thermal contributions to PLL deformation. CD data were collected at 1 min intervals at each
shear rate using a data interval of 0.1 nm, a bandwidth of 1.0 nm, response time of 4 s, and a scanning speed of 100 nm/min. Solvent spectrum was used as baseline for data collection.

*Calculations of helix content:* The helix content of the four PLL chains were calculated based on methods by Chen *et al.* (262). By applying the chain-length-dependent model, the fractional helicity ($f_h$) or helix content of individual PLL chains was estimated using the mean residue ellipticity at 222 nm, $[\theta]_{222}$, according to the following function (262, 263):

$$f_h = \frac{[\theta]_{222}}{[\theta_{\infty}]_{222} \left(1 - \frac{k}{n}\right)} \times 100\%$$  \hspace{1cm} (7.1)

where $[\theta_{\infty}]_{222}$, given as -39500 deg.cm$^2$dmol$^{-1}$, is the mean residue ellipticity of an ideal peptide with 100% helicity, $n$ is the number of residues per helix, and $k$ (chain-length-dependent factor) is a wavelength dependent constant (2.57 at 222 nm).

**7.3 Results**

The solution conformation of the four PLL samples were studied *in situ* for the selected shear rates over a period of 1 h. Under quiescent conditions, the CD spectral features of the four PLL samples, at 20°C, were consistent with a classic $\alpha$-helix structure with two minima at 209 and 222 nm. However, the magnitude of the negative intensity in the $\alpha$-helix region was progressively higher with increasing chain-length, indicative of a growing helix content (figure 7.1). Consistent with this observation, quantitative calculations showed an increase in the helix content of PLL as a function of chain-length (figure 7.2). For example, whereas the helix content of the highest molecular weight (381.2 kDa) was 93.6%, that of the shorter chains (<205.7 kDa) contained significant amounts of random coils and turns.
Figure 7.1: Chain-length dependence of the CD spectra of PLL. The mean residue ellipticity (MRE) of the PLL chains increases as a function of chain length. The wavelength scans were collected for (○) 15 kDa, (●) 68.3 kDa, (◊) 205.7 kDa, and (♦) 381.2 kDa.

Figure 7.2: Chain-length dependence of the helix content of PLL. The figure shows that the helical content of PLL increases as a function of molecular weight. The error bars represent ± standard deviation.
While the PLL samples were stable under quiescent conditions over the 1 h period, structural changes in the α-helix region were observed upon exposure to shear. Representative CD spectra from the 205.7 kDa sample (figure 7.3) show that at the onset of shear, the intensity of the negative ellipticities at 209 and 222 nm diminish as a function of time, indicating a decline in the helix content. The observed change was more pronounced with increasing shear rate. For example, at a relatively low shear rate (117 s$^{-1}$), there was no significant change in the spectral features of the wavelength scans. At the highest shear rate (715 s$^{-1}$), however, a sharp decline in the two helix minima at 209 and 222 nm was observed. In addition, an isodichroic point in the region of 203 nm was observed in the high shear regime, which suggests that the shear-induced unfolding of the α-helical PLL structure follows a two-state transition. This observation was substantiated by further analysis of the conformational transitions using phase diagrams (figure 7.4). The phase plots were generated using the negative ellipticities at θ$_{204\text{nm}}$ and θ$_{222\text{nm}}$. Clearly, the two linear segments in the plot for the high shear regime confirm the occurrence of a two-state conformational transition, and the existence of at least one intermediate state during the unfolding of the α-helix-PLL structure. It is noteworthy that the longer chain molecule (381.2 kDa) showed no such conformational transitions as the extent of unfolding was minimal even at 715 s$^{-1}$.

The relationship between shear rate and the decline in negative ellipticity at 222 nm is shown in figure 7.5 using representative data from the 205.7 kDa sample. The kinetic graphs were obtained by plotting the ratio of the time-dependent change in the 222 nm minimum as a function of shear rate. The molar ellipticity value at 222 nm is widely used as a diagnostic for the α-helix structure (205). Clearly, as the magnitude of the shear rate increased, with the consequent increase in the hydrodynamic drag on the PLL chains, a sharp decline in the helix content was observed. The observed change was irreversible, which is possibly because the unfolding PLL chains get trapped in a metastable conformation that is prone to aggregation.
Figure 7.3: CD spectra of the 205.7 kDa PLL sample in simple shear flow. The sample was exposed to shear rates of (A) 117 s\(^{-1}\) and (B) 715 s\(^{-1}\) for 1 h. The spectra shows wavelength scans from zero (no shear) to 60 min at 5 min intervals.
Figure 7.4: Phase plot of the theta values at $\theta_{204}$ nm and $\theta_{222}$ nm using data from figure 7.3B. The figure shows two linear segments indicative of a two-state conformational transition with at least one intermediate.

Figure 7.5: Change in the relative helix content of PLL (205.7 kDa) as a function of time. The shear rates applied were: (□) 74 s$^{-1}$; (●) 117 s$^{-1}$; (◊) 219 s$^{-1}$; (▼) 302 s$^{-1}$; (○) 518 s$^{-1}$; and (▲) 715 s$^{-1}$. The figure shows that the unfolding of the $\alpha$-helix-PLL structure depends on both the magnitude of shear and the duration of its application. The error bars represent $\pm$ standard deviation.
The influence of chain-length on the shear-stability of the PLL molecules was investigated by comparing the extent of unfolding of the α-helix-PLL structure, after 1 h of shear exposure, for the four molecular weights as a function of shear rate (figure 7.6). The plot was generated by calculating the percentage change in the helix content of individual PLL chains, taking the initial helix content per chain under quiescent conditions to be 100%. It is important to note that the estimated helical content of the unsheared PLL is a function of molecular weight as shown in figure 7.2. Here, the extent of unfolding of the α-helix-PLL structure in simple shear flow was found to also depend on the chain-length of the PLL molecules. For example, at 715 s⁻¹, only ~24% of the initial helix content of the 381.2 kDa sample unfolded compared to ~80% in the shortest chain (15 kDa). The data suggest that at a given shear rate, the shorter chain PLL molecules unfold more rapidly in the flow field relative to their longer chain counterparts. In addition, the extent of unfolding was more pronounced with increasing shear rate for all molecular weights, approaching equilibrium beyond 500 s⁻¹.

Figure 7.6: Molecular weight dependence of the extent of helix unfolding in shear flow. Deformation of the α-helix-PLL structure was less pronounced with increasing molecular weight: (◊) 15 kDa; (●) 68.3 kDa; (□) 205.7 kDa; and (▼) 381.2 kDa. The error bars represent ± standard deviation.
The data was further analysed by considering the molecular weight dependence of the helix content of the PLL chains at a given shear rate (figure 7.7). The highest shear regime, where the unfolding of the α-helix-PLL structure was more pronounced, was considered. The data shows a strong linear correlation of the percentage change in helix as a function of molecular weight. Furthermore, the data clearly reveals the hysteresis in the long chain helices even in the highest shear regime applied.

Figure 7.7: Molecular weight dependence of the helix content after 1 h of shearing at 715s⁻¹. The broken line is a linear fit to the data with an R² value of 0.99. The error bars represent ± standard deviation.

Interestingly, using representative data from the 68.3 kDa sample, a plot of the change in helix content against shear strain (γt) also showed a strong correlation, typical for the four molecular weights studied (figure 7.8). From this plot, the unfolding transition of the α-helix-PLL structure occurred at a strain value in the region of 10⁵, and was found to be independent of the chain-length. By introducing the solvent viscosity, η (10³ Pa.s), a similar trend is observed if the change in helix content were plotted against the cumulative stress (ηγt) (i.e. stress history). Here, the critical cumulative stress (τc) value
for the deformation of the PLL helix structure is of the magnitude $10^1$ Pa.s. In addition, by taking the time point where the two linear segments intersect in the phase diagram (figure 7.4), a similar strain value is observed. This clearly shows that the conformational transition in the sheared PLL chains indeed begins at this strain value.

![Figure 7.8: Change in the helix content of PLL as a function of shear strain. The figure shows that the $\alpha$-helix-PLL structure (68.3 kDa) is stable below a strain value of $10^5$. For clarity, the shear rates plotted are: (□) 74 s$^{-1}$; (◊) 302 s$^{-1}$; (○) 518 s$^{-1}$; and (●) 715 s$^{-1}$, and error bars representing $\pm$ standard deviation are shown only for the sample sheared at 518 s$^{-1}$.](image)

### 7.4 Discussion

Studies of the shear-stability of dilute protein systems, and polymers at large, is of immediate relevance to both industry and medicine. The complexity of this subject has resulted in a greater number of theoretical studies, rather than experimental investigations, to appreciate the conformational dynamics of polymers in shear flow (24-26, 125). The handful of experimental studies on protein systems in this field gives conflicting reports (6, 27, 59, 84). These differences can be ascribed to a number of factors including the type of flow applied, the duration of shear exposure (residence
time), and solvent viscosity. More importantly, differences in the primary structure of protein systems have been found to influence their shear-stability(233), which further complicates data interpretation and extrapolation to other systems. The current study presents an ideal case where the unfolding dynamics of different molecular weights of the same system are compared, with the aim of understanding the shear-stability of an α-helical structure in shear flow. Needless to say, the α-helical structure is common to many peptides and proteins, and the structural destabilization of a native protein conformation is a prerequisite for aggregation and amyloid fibril formation (14).

*Chain length dependence of $[\theta]_{222\text{nm}}$ (helix content)*

At high pH (pH > 10), the lysyl residues making up poly-L-lysine are neutralized in solution. The α-helical conformation assumed by the homopolypeptide, below 30°C, is attributable to intramolecular hydrogen bonds between lysine residues, and in the absence of electrostatic interactions, is primarily stabilized by intramolecular hydrophobic interactions (264). Theoretical studies suggest that, in addition to a stabilizing solvation effect, attracting forces within an α-helix provide extra stability to the overall helix structure (265). Thus, an increase in chain-length, with the consequent increase in α-helical turns, confers additional stability on a PLL chain (266). However, it is argued that a dipole moment induced clustering of helices in high molecular weight PLL may exclude hydrating water molecules from the inter-helical space, leading to a possible helix destabilization (149). This feature was purported to explain the thermal susceptibility of long PLL chains in the α-helix state. However, extension of polyalanine chain-length was found to favour α-helix conformations even in a hydrophobic environment (265).

The CD spectra of the four PLL molecular weights studied show α-helix secondary structural features at elevated pH. This was expected since the supplier reported degree of polymerization for the shortest chain (15 kDa by viscosity), given as 71, is sufficient for the formation of secondary structures such as α-helix and β-sheet. It is generally accepted that the molar ellipticity value at 222 nm ($[\theta]_{222}$) is directly proportional to the number of residues in a helix (262). However, this value is found to be influenced by the chain-
length and dynamic motion of a polypeptide chain (205). That is, longer chain helices show a higher negative ellipticity at 222nm relative to their shorter chain counterparts. This explains the observed increase in $[\theta]_{222}$ as a function of molecular weight. Hence, by using the length dependent model (equation 7.1), which takes into account the end-effects of longer helices, it was not surprising that the calculated helix content increased with increasing chain-length. A simple explanation for this observation is that, as the PLL length grows, an increasing number of the lysine residues form part of the helical segments. Indeed, the helicity and stability of synthetic peptides were found to increase with increasing chain-length (266). The conformational attributes of the four PLL chains studied, under quiescent conditions, are summarized in table 7.1.

Table 7.1: Key properties of the PLL chains in the quiescent state as determined from the helix dimensions.

<table>
<thead>
<tr>
<th>$M$ (kDa)</th>
<th>DP</th>
<th>H (%)</th>
<th>$H^a$</th>
<th>$C^a$</th>
<th>helix turns$^b$</th>
<th>$H^c$</th>
<th>$C^c$</th>
<th>$L^d$ (nm)</th>
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<td>55.2</td>
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<td>27.1</td>
<td>134.3</td>
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<td>1707.3</td>
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</tbody>
</table>

DP is the supplier reported degree of polymerization by viscosity. H and C represent the helix and coil conformations of native PLL chains. $^a$Number of lysine residues in a particular conformation. $^b$Number of helix turns per chain assuming that one turn involves 3.6 Lys residues. $^c$Total length (nm) of the helix and coil segments of the PLL chain assuming a helix pitch of 0.54 nm and the length of a Lys residue in the PLL chain to be 0.917 nm.(2) $^d$Combined length of the PLL chain.

**Unfolding of poly-L-lysine molecules in simple shear flow**

Like other polymers, poly-L-lysine chains exposed to simple shear flow are expected to undergo a combination of rotational and extensional dynamics, dictated by the flow field, even at moderate shear rates (24, 267). In the extensional flow field, the molecules experience a hydrodynamic drag which counters the cohesive forces stabilizing the native
α-helix conformation. Theoretical studies suggest that the frequency of stretching events, hence average polymer extension, in simple shear flow amplifies with increasing shear rate (58). This is not surprising since an increase in shear rate imparts a stronger hydrodynamic drag to the flow field. In contrast, the fluid forces acting on PLL molecules recede as the molecules orient in the rotational component of the flow field. As individual molecules randomly access the two flow fields, temporal fluctuations which involve periodic elongation, relaxation and tumbling occur (58, 247). For this reason, sharp conformational transitions, such as helix-to-coil, are not expected in simple shear flow (24). This is consistent with molecular dynamics simulations which reveal that, unlike pure extensional flow, the coil-stretch transition in simple shear flow involves intermediate states (24, 124).

The four PLL molecular weights studied showed a time- and shear rate-dependent decline in the negative molar ellipticity value at \([\theta]_{222}\), which clearly shows a loss in helicity. Unfolding of the α-helix-PLL structure is expected to occur when molecules are aligned in the extensional flow field and the magnitude of the hydrodynamic drag overwhelms the restoring (cohesive) forces stabilizing the PLL chains. At lower shear rates, only moderate losses in helicity occurred. In high shear regimes, a two-state conformational transition was observed in the PLL chains except for the heaviest chain (381.2 kDa), where the change in the helix content (\([\theta]_{222}\)) was minimal. This is consistent with the notion that the hydrodynamic drag is enhanced as the shear rate increases. The existence of intermediate states during PLL unfolding is also consistent with theoretical predictions. On comparing the extent of structural deformation in the four molecular weights, the flow-induced unfolding of the α-helix-PLL structure was found to be less pronounced with increasing chain-length. In fact, in the highest shear regime, a strong linear correlation was observed between the percentage change in helix and chain-length after 1 h of shearing. This implies that the shorter chain helices unravelled more rapidly with increasing shear rate relative to the long chain helices.

Although a number of theoretical studies predict the helix-coil transitions in polymers, especially homopolypeptides, under stress, little work has been expended on the
molecular-weight-dependence of this phenomenon (268-270). To explain the observed trends, it is important to invoke simple theory regarding polymer dynamics in shear flow. Firstly, the PLL chain is considered as a rod-like structure. Here, the hydrodynamic forces on the rod will increase with both molecular weight and shear rate. Following the arguments of Bruinsma (274) for the stress on a rod of “hydrodynamic beads,” the stress on the helix is derived as:

$$\tau = \frac{3}{16} \pi \eta \gamma L^2 \sin 2\theta$$  \hspace{1cm} (7.2)

where $\eta$ is the solvent viscosity, $\gamma$ the shear rate, $L$ the length, and $\theta$ the angle subtended to the flow direction. As the length $L$ is directly proportional to the molecular weight, the strain will be proportional to $M^2$ and the remaining helix, $\alpha$, will be inversely proportional to the strain. That is, $\alpha \sim M^{-2}$. Therefore, classical theory would predict that the deformation (unfolding) of PLL chains increases with increasing chain length. This is not observed in the results presented, which show an $\alpha \sim M$ curve (figure 7.6). Therefore, there must be an alternate explanation for the observed trends.

In order to understand the data, a second classical approach is considered. The PLL chains are assumed to form blobs in a theta solvent, and hence exists as collapsed ($\alpha$-helix) globules under the experimental conditions used. Here, it is assumed that the “blobs” are formed by folded helices which interact in a manner resembling an associative network. The blob, then an object of elastic nature which may be modelled using classical “rubber elasticity” (275). The shear modulus or modulus of rigidity ($G$), which in this case describes the response of PLL chains to hydrodynamic deformation/strain ($\gamma$) is given as: $G = n k T = \frac{\tau}{\gamma}$, where $n$ is the number of interactions per unit volume. By incorporating the volume of a spherical globule, the shear modulus can be re-written as: $G = \frac{3kT}{4\pi r^4 M^{1/2}}$. The molecular weight dependence of the radius of a spherical globule has been assumed to be $r = aM^{1/2}$. Clearly, the rigidity of the PLL molecules decreases with increasing chain-length. In the flow field however, the
hydrodynamic drag \( f_{\text{hyd}} \) and stress \( \tau \) experienced by the PLL chains are

\[
f_{\text{hyd}} = 6\pi \eta \dot{\gamma}
\]

and

\[
\tau = \frac{2}{3} \eta \dot{\gamma}
\]

respectively. A combination of the hydrodynamic stress and shear modulus gives the following scaling relationship:

\[
\gamma \propto \frac{1}{\alpha} \propto M^{1/2}
\]  \( (7.3) \)

Here, it is noted that the amount of deformation \( \gamma \) varies inversely with the helix remaining \( \alpha \) after shear exposure. Therefore, both classical interpretations would predict that the deformation (unfolding) of the PLL chains increases with increasing chain-length, which is inconsistent with the experimental results. The key experimental results are summarized as follows: (i) \( \alpha \propto (\gamma \dot{t})^{-1/2} \); (ii) \( \alpha \propto M \); and (iii) \( \gamma \dot{t}_c \approx 10^5 \), where \( \gamma \dot{t}_c \) is the critical strain.

More recently, theoretical studies of the dynamic instabilities of collapsed polymers in simple shear flow provide some simple scaling arguments worth noting (25). By taking into account the opposing effects of the fluid drag and the cohesive forces stabilizing a polymer chain, the authors predict the monomer size dependence of the critical strain rate required to induce unfolding by the following scaling relationship:

\[
\dot{\gamma}_c \propto R
\]  \( (7.4) \)

where \( R \) is the radius of the collapsed globule. Here, the critical strain rate, required for deformation/unfolding, varies linearly with the hydrodynamic radius of a given polymer chain (25). Hence, it is expected that shorter PLL chains would show shear-instabilities at relatively low strain rates compared to the longer chains. This is explained by the phenomenon of hydrodynamic screening, where the hydrodynamic interactions between lysyl side chains leads to a reduction of the fluid shear rate and hence the fluid forces acting on the surface of individual PLL globules. In addition, only the outer segments of the globules are directly exposed to the fluid drag in the flow field (24). Therefore, as \( R \) grows, the critical shear rate required to unfold the PLL globule increases. However, the hydrodynamic interactions between lysyl side chains are reduced once the globule begins
to unravel, leading to rapid unfolding, especially in a purely elongational flow field (24, 276).

Simply put, at a given shear rate, enough to initiate unfolding, it is expected that short-chain PLL helices will unfold more rapidly in the flow field. This conclusion is further buttressed by computational predictions of the chain-length effect of dilute polymer solutions in elongational flow (273). The authors observed that relative to shorter chains, longer chains remain in a coil-like state for larger strains. A schematic depicting this conclusion is shown in figure 7.9. This figure illustrates that whereas the short chain helices stretch easily in the extensional flow component of the flow field, the long chains show greater shear-stability.

Figure 7.9: Schematic of the shear-induced unfolding of the α-helix-PLL structure in simple shear flow. The figure illustrates a rapid unfolding of short chain helices upon shear exposure, whereas the long chain helices experience structural distortions with minimal unfolding.
A priori, it is reasonable to ascribe the hysteresis observed in the long chain PLL to the greater stability of the helix-structure, originating from the large network of hydrophobic interactions in concert with attracting forces within the helices (172, 266). Hence, even at 715 s\(^{-1}\), the restoring force in the 381.2 kDa sample counterbalance the fluid drag in the extensional flow cycle and only ~24% of the initial helix content is lost over the 1 h period. In fact, de Gennes notes that in flow fields such as simple shear flow, where the coil-stretch transition is discontinuous, hysteresis should often be observed (24). In addition, the compact folding of the long PLL chain, perhaps giving rise to a tertiary configuration, provides hydrodynamic screening for the hydrophobic helical segments concealed in the PLL matrix (24, 25, 119, 274). Hence, only the outer segments are exposed directly to the flow field. This, in effect, reduces the hydrodynamic drag acting on the entire chain, leading to a minimal chain stretching in the extensional flow field. Although dilute PLL samples were used in this study, the possibility of tangling of partially extended conformations during the periodic end-over-end tumbling in the flow field cannot be ruled out. The occurrence of this event may give rise to conformational distortions resulting in kinked states (272, 275), especially at high shear rates, which may slow the unravelling of the PLL helix. This in turn may enhance intermolecular hydrodynamic interactions, providing additional hydrodynamic shielding of helical segments from the fluid drag. In fact, theoretical studies(24) and molecular dynamics simulations(128) predict the opposing effect of hydrodynamic interactions to protein unfolding in both uniform and elongational flow fields.

It is interesting to note that the four PLL molecular weights gave a similar strain value of \(10^5\), below which the helix structures were intact, although the extent of unfolding was less pronounced with increasing molecular weight. The data reveal that although the intrinsic properties of the PLL helix are independent of chain-length, its shear-stability arises from the hydrodynamic screening effect of the long chains. More importantly, the data suggest that the strain rate is not as critical as the duration of its application. That is, provided the PLL chains, irrespective of the molecular weight, are exposed to a fixed shear rate for a sufficient amount of time, the helical segments will eventually unfold at a critical strain value \(\geq 10^5\). This makes the idea of a critical shear rate only arbitrary. In
fact, a similar shear strain value had previously been reported for globular proteins of varying molecular weights (6, 7).

Proteins present a more complex network of intra-/intermolecular interactions compared to a homopolypeptide like poly-L-lysine. However, a similar shear effect on the helical structures of bovine insulin, in the preceding chapter, and bovine serum albumin(74) even at a moderate shear rate of 300 $s^{-1}$ have been observed. In the insulin sample, fibrillar species were observed in high shear regimes even at room temperature. Indeed, several studies show that shear flow enhances amyloid fibril formation (54, 107, 112, 113). Hence, if this observation pertains to the majority of protein systems, the shear effect could enhance the formation of β-sheet structures under favourable solution conditions such as elevated concentrations and temperature. This has serious implications in amyloid related diseases and vascular disorders, as well as quality control during the commercial isolation and purification of protein products.

7.5 Conclusion

The α-helical-PLL structure unfolds in simple shear flow. The extent of unfolding is dependent on the monomer size of the PLL chains, as well as shear rate and the duration of its application. The shear-stability of the α-helical-PLL structure increased with increasing chain-length. However, the shear strain value of $10^5$, required to unfold the α-helical-PLL structure, was independent of the molecular weight of PLL. The following dependence of the remaining helix ($\alpha$) was observed: (i) $\alpha \propto (\dot{\gamma}t)^{-1/2}$ for all molecular weights measured and for strain values above the critical value $\dot{\gamma}t_c \approx 10^5$; and finally (ii) $\alpha \propto M$ where the remaining helical content is proportional to the molecular weight.
8. Overview

Protein molecules are subjected to potentially denaturing fluid shear forces during processing and in physiology. For this reason, the stability of protein molecules in hydrodynamic flows has received considerable attention in the literature, but remains to be fully understood. Preliminary researchers in this field were limited by techniques that lacked the sensitivity and time resolution to directly monitor the structural changes in protein solutions exposed to hydrodynamic flows. Recent techniques, including fluorescence spectroscopy, have overcome these challenges, but are confronted by the inability of those techniques to provide quantitative information on the conformational dynamics of protein molecules in shear flow. The main aim of this thesis was to investigate the stability of selected peptide/polypeptide systems in uniform and heterogeneous hydrodynamic flows. A combination of qualitative and quantitative structural techniques was employed to study the conformational dynamics of the selected systems in situ and in real time.

Preliminary studies in this work considered probing protein deformation and aggregation using intrinsic protein fluorescence (chapter 4). Bovine insulin was used as the model system. On comparing the kinetic profiles of ThT, ANS, light scattering, and intrinsic Tyr fluorescence during insulin fibrillation, it was demonstrated that the sequence of structural changes (dimers → monomers → partially unfolded monomers → oligomeric aggregates → fibrils) accompanying the aggregation process can be detected directly using intrinsic Tyr fluorescence. The sensitivity of this technique is complementary to the use of extrinsic fluorescent probes in protein denaturation/aggregation studies. In addition, obtaining such critical information from the protein itself, via its intrinsic fluorescence, affords the advantage of directly examining structural changes that occur at the molecular level; providing concrete details of the early events preceding aggregation. Furthermore, the aggregation kinetics can be monitored in the absence of extrinsic fluorescent probes, which excludes the possible contributions of these probes to the aggregation process. More importantly, this technique was instrumental in performing fluorescence studies of the shear-stability of bovine insulin in Couette flow (chapter 6).
The stability of peptide/polypeptide systems were studied in two flow regimes: (i) heterogeneous flow, which is characterized by a non-uniform velocity gradient with variations in hydrodynamic shear stress in time and space; and (ii) uniform flow (Couette flow), which is characterized by a homogeneous velocity gradient with a consistent hydrodynamic drag in time and space. It was demonstrated that the irregular flow patterns and mixing associated with heterogeneous flow regimes greatly enhance the aggregation of amyloid-β into mature fibrils (chapter 5). It was revealed that shearing triggered the nucleation of oligomeric aggregates, which serve as the building blocks for Aβ fibrillation, and facilitated the polymerization of these aggregates into mature fibrils.

To gain additional insight into the influence of fluid shear stress on protein solutions, further studies were performed in a well defined flow field (Couette flow). It was demonstrated that hydrodynamic drag triggers the unfolding of the helical segments of bovine insulin upon exposure to shear flow. The study employed both qualitative and quantitative structural techniques, fluorescence and circular dichroism respectively, to monitor the structural instabilities in situ and in real time during shear flow. The shear-effect was more pronounced with increasing shear rate and the duration of its application. Morphological analysis of the sheared samples revealed that denatured insulin molecules coalesced into oligomeric aggregates in relatively low shear regimes, and polymerized into protofibrillar aggregates in high shear regimes. Further studies with poly-l-lysine, commonly used as a model protein system, substantiated the observation that fluid drag can unfold the helical segments of a natively folded polypeptide chain. A molecular-weight-dependence of this phenomenon was observed on comparing the percentage change in helix content of PLL as a function of chain-length. It was observed that although the four molecular weights showed a critical strain value, $\sim 10^5$, the extent of unfolding was less pronounced with increasing chain-length. This observation contradicts classical polymer physics theories, but is in tune with recent models which propose the stability of longer polymer chains in shear flows via screening of chain segments from the fluid drag. Collectively, it was demonstrated that fluid forces can trigger structural instabilities in natively folded proteins leading to the formation of aggregates and/or amyloid fibrils.
Given that the partial unfolding of natively folded proteins is a prerequisite for aggregation, this work highlights an important parameter to be considered in the quest to unravel the conditions that promote protein denaturation and aggregation both \textit{in vivo} and \textit{ex vivo}. Future studies in this field should consider performing similar experiments in elongational flow fields, and include an array of polypeptides for a broader assessment of the shear-effect on protein systems. However, it important to appreciate the difficulties associated with the various types of equipment used to generate elongational flows, as regards the range of conditions and the sorts of optical measurements that can be applied.
9. References


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