Flinders University

College of Science & Engineering

Engineering Thesis ENGR9700A

Develop new technologies for protein structure manipulation and monitoring to better understand amyloidosis diseases

(Thesis submitted to the College of Science and Engineering in partial fulfilment of the requirements for the degree of Master of Engineering (Biomedical) at Flinders University, Adelaide, Australia)

Name: Haozhen Hu

Student ID number: 2194142

Supervisor: Youhong Tang

Submission Date: 22/10/2020

Declaration:

I, Haozhen Hu, hereby declare that the thesis entitled 'Develop new technologies for protein structure manipulation and monitoring to better understand amyloidosis diseases' is entirely my original work for the award of Master of Biomedical Engineering. I have completed this study under the guidance of Prof Youhong Tang, Flinders University, Collage of Science and Engineering.

I also declare that this thesis has not been submitted for any degree in any university previously.

2nd October 2020

Abstract:

This thesis aims to manipulate and monitor protein unfolding/refolding for contributing to the potential development of amyloidosis disease treatment and detection. Due to the low molecular weight and comparatively simple structure, protein beta-lactoglobulin is chosen in this study. Manipulation of the protein folding state is accomplished by using the vortex fluidic device (VFD) and high concentration of chaotropic agent guanidine hydrochloride (GuHCl), comparing to the traditional ways of refolding protein, VFD has the advantages of low cost and easy to control (from rotation speed or tilt angle). For the protein structure detection and monitoring, the conventional way by using circular dichroism (CD) and the novel way by using aggregation-induced emission (AIE) fluorescence are applied. Among them, fluorescence is believed to be relatively cheaper and giving information more directly so it is more convenient for this usage, moreover, the dye of AIE fluorescence shows great reactivity and sensitivity.

The project starts with establishing analyzing protocol, the lower negative ellipticity at 216nm of CD measurement and higher fluorescence intensity at 470nm indicate larger protein denaturation extent. The experimental parameter settings include chemical concentrations, reaction time, and VFD settings. Since protein concentration and VFD settings have been determined in the previous studies, the denaturant concentration 4M and AIE labelling time 2.5 hours are investigated and used in this project.

VFD was performed for both refolding and unfolding experiments, the results show that VFD can effectively refold the unfolded protein, as well as boost the reaction of denaturant and protein to unfolded protein. A combination experiment is designed to demonstrate the conditions for those two processes, which further proves that when denaturant is present in the solution, the two mechanisms would both existed, however, denaturation would still be dominant. To have a reference for the analysis result of the CD data, DichroWeb is introduced in this project, which is an online analysis tool for protein circular dichroism spectra, a convincing result has been obtained.

Finally, future works are proposed for optimizing the experimental result and avoiding the error caused by uncertainty factor, including the fluid behavior modification by changing VFD rotational speed and the fluorescence blue shift due to disordered protein aggregation.

Acknowledgement:

Conducting a research and writing a thesis is far more difficult than I thought and more rewarding than I could have ever imagined. First and foremost, I would like to express my sincere gratitude to my supervisor Prof. Youhong Tang for the continuous support of my Master degree research and thesis, for his dynamism, vision and motivation which has deeply inspired me. I learn from him more than just how to conduct this project, but also the logic flow, methodology and the way to communicate and present to others for any project. It was a great privilege and honor to work and study under his guidance. I am extremely grateful for what he has offered me.

Besides my supervisor, I would like to extend my sincere thanks to research assistant Miss Xinyi Zhang, who patiently helped me and spared no effort to offer me suggestion throughout the whole year, it is your motivation and sincerity that inspired and encouraged me. I would like to thank Dr. Xuan Luo for offering me the training and giving me suggestion on how to use VFD, also providing support when I wanted to try using fiber optics. Many thanks to Dr. Yuning Hong for giving me advises every fortnight on the group meeting, especially pointing out that the fluorescence will keep once the AIE dye bonded with unfolded protein, even when the proteins are refolded, it is a very important part in my experiment.

My completion of the project and thesis would not be accomplished without Dr. Shouxiang Zhang for kindly offering me the AIE dye, Dr. Carlie Delaine and Dr. Andrew Blyth for patiently providing me training and supporting on using CD machine, and Dr. Bonnie Wallace for permitting my application of DichroWeb using.

Table of Contents

Declaration: II

Abstract: III

Acknowledgement: IV

Chapter 1: Introduction: 1

1.1 Background 1

1.2 Vortex Fluidic Device 1

1.3 Measurements 3

1.3.1 Circular Dichroism 3

1.3.2 Aggregation-induced Emission Fluorescence 4

Chapter 2: Literature review: 6

2.1 Aggregation-induced Emission 6

2.2 AIE detecting protein folding state 6

2.3 Amyloidosis 7

2.4 Circular dichroism measuring protein structure 7

2.5 Technologies of refolding protein 8

2.6 Vortex Fluidic Device 9

Chapter 3: Materials: 9

Chapter 4: Method and Result: 10

4.1 Experiment preparation 10

4.2 Establishment of fluorescence signal...11

4.3 Establishment of CD spectrum 13

4.4 AIE labelling time...14

4.5 Determining the concentration of GuHCl...15

4.6 Refolding protein using VFD 17

4.6.1 CD measurement 17

4.6.2 FL measurement 19

4.7 Using VFD during denaturation 20

4.7.1 CD measurement 20

4.7.2 FL measurement 21

4.8 The verify experiment...22

4.9 Analyzing the CD result from DichroWeb..24

Chapter 5 Future work 28

5.1 VFD rotational speed...28

5.2 Blue shift of FL intensity peak 31

Chapter 6: Discussion:...34

Chapter 7: Conclusion: 37

References: 38

Table of figures:

Figure 1 VFD 2

Figure 2 CD result of α-helix, β-sheet and random coil 4

Figure 3 The mechanism of TPE-MI reacts with unfolded protein 5

Figure 4 Fluorescence of denatured protein, fluorescence of TPE-MI, fluorescence of 3h denatured protein + TPE-MI and fluorescence of 24h denatured protein + TPE-MI 12

Figure 5 The CD results of undenatured protein, 3h denatured protein and 24h denatured protein 13

Figure 6 The fluorescence intensity change during AIE labelling 15

Figure 7 Fluorescence intensity of using different concentration of GuHCl 16

Figure 8 The CD result of after 24h denatured protein and after 24h denatured protein + VFD.. 17

Figure 9 A. The fluorescence result of after 24h denatured protein and after 24h denatured protein + VFD. B. The error bar of experiment 9 19

Figure 10 The CD result of after 3h denatured protein and after 3h denatured protein + VFD.. 20

Figure 11 The fluorescence result of after 3h denatured protein and after 24h denatured protein + VFD 21

Figure 12 The verify experiment (Fluorescence).. 23

Figure 13 DichroWeb home page 25

Figure 14 The analysis of Figure 9A obtained from DichroWeb. A. The protein solution after 24 hours denaturation without using VFD. B. The protein solution after 24 hours denaturation combining VFD......26

Figure 15 The analysis of Figure 11 obtained from DichroWeb. A. The protein solution after 3 hours denaturation without using VFD. B. The protein solution after 3 hours denaturation and VFD......27

Figure 16 The fluorescence of VFD rotational speed 4000rpm and 5000rpm in refolding experiment......29

Figure 17 The fluorescence of VFD rotational speed 3000rpm, 4000rpm, 5000rpm and 6000rpm in unfolding experiment......30

Figure 18 The fluorescence of using 4 days preserved β-lactoglobulin......32

Figure 19 The fluorescence of using 7 days preserved β-lactoglobulin......33

Chapter 1 Introduction:

1.1 Background

In the human body, protein folding is the process by which a protein obtains its functional structure and conformation. Through this physical process, proteins fold from irregular folding to a specific functional three-dimensional structure. Eventually, the body reaches a state called proteostasis which represents a normal ratio of folded protein and unfolded protein. While in some cases such as gene mutant, the proteins can misfold and aggregate, and finally break the state of proteostasis. The aggregation of misfolded protein is called amyloid. The diseases associated with amyloids are known as amyloidosis, the common amyloidosis includes Alzheimer's disease, Parkinson's disease, and Huntington's disease.

Taking Huntington's disease (HD) as an example, it is an autosomal dominant hereditary neurodegenerative disease, discovered by the therapist George Huntington in 1872. The main cause of it is the fourth of chromosome in patients with Huntington gene mutates, leads to the variation of protein, the protein in cells gradually gathered to form a large molecule accumulate in the brain, affect the function of nerve cells. The disease usually begins in middle age and is characterized by dance-like movements. As the disease progresses, the patient gradually loses the ability to speak, move, think, and swallow. The disease lasts about 10 to 20 years and eventually leads to death. According to the statistics, in Australia, over 1800 people have HD and approximately 9000 are at risk.

1.2 Vortex Fluidic Device

Studying of protein refolding in vitro can be greatly contributing amyloidosis treatment. The currently used protein refolding in vitro process can be divided by three steps: First, isolation of inclusion bodies through low speed centrifugation of bacterial cells, which are already mechanically disrupted by ultrasonication, French press or high-pressure homogenization. Inclusion body is an organization in body cells where the misfolded proteins directly go into, it usually contains very little host protein, ribosomal components or DNA/RNA fragments and mostly overexpressed proteins. Secondly, solubilization of inclusion bodies by using high concentrations of chaotropic agents to completely denature the protein secondary structures and result aggregation protein molecules. Thirdly, protein refolding, methods include direct

dilute, membrane controlled denaturant removal, chromatographic and hydrophobic interaction chromatography. These methods have the common disadvantages of time-consuming and hard to control the process.

Vortex Fluidic Device (VFD) is a device that can create a dynamic thin fluid film, by providing high shear stress, high shearing rates, rapid heat and mass transfer, micromixing and fluidic pressure waves, this device has been proven the great performance in biocatalysis, organic synthesis, materials synthesis and probing self-organised systems. Furthermore, it is reported able to refold proteins, and get rewarded for ‘A chemical recipe to partially unboil an egg’ in 2015 Ig Nobel chemistry prize. The slogan of Ig Nobel prize is ‘first let you laugh, then let you think’. In this case, people would laugh about the idea of making a cooked egg become a raw egg but will think the great potential of protein renaturation using VFD technology.

Removed due to copyright restriction

Figure 1 VFD

Parameters of VFD include the crucial one of rotational speed, tilt angle and rotational direction (CW or CCW). The current study indicates that the different rotational speed will change the way of the fluid present within the tube, also it will be changed between different kinds of fluid. For the tilt angle, it is proven that the angle of 45 degrees between the tube and the ground is the most efficient angle for any purpose of VFD using. For the rotational direction, obviously using the opposite direction in one experiment would cause some unwanted outcomes, even though the current research has not drawn a comprehensive conclusion, but it is recommended to stick with one direction in one research.

Temperature can be a major variable when using VFD since the high rotation speed of the VFD tube will obviously result in the temperature change of the inside fluid. According to the research, when using VFD at 5000 rpm the maximum temperature change is three degrees, in this project, the effect is neglected.

1.3 Measurements

1.3.1 Circular Dichroism

It is well known that protein has four levels of structure while it is in the folding process. Protein primary structure is a sequence of a chain of amino acids, the individual amino acids join in the long chains by forming peptide bones, sequences with fewer than 50 amino acids are generally referred to as peptides; protein secondary structure is where the protein local folding of the polypeptide chain into helices or sheet, the main force is hydrogen bonding, α-helix is a right-handed coiled strand and β-sheet is the pairs of strands lying side-by-side; protein tertiary structure is the three dimensional folding pattern of a protein due to side chain interactions, the protein molecule will bend and twist in such a way as to achieve maximum stability or lowest energy state; and protein quaternary structure is the protein consisting of more than one amino acid chain. According to the definition of the protein structure, it is believed that protein folding mainly happens on protein secondary structure and protein tertiary structure.

Circular dichroism (CD) is a useful technique when measuring protein secondary structure and protein tertiary structure. The principle of it is it uses circularly polarized light, optically active chiral molecules have their preference absorbing one direction of circularly polarized light. Due to different absorption of a protein to left-hand circular polarized light (LHC) and right-hand circular polarized light (RHC), the circular dichroism of the protein can be shown as ellipticity over a range of wavelength. Furthermore, the information on protein secondary structure can be gotten.

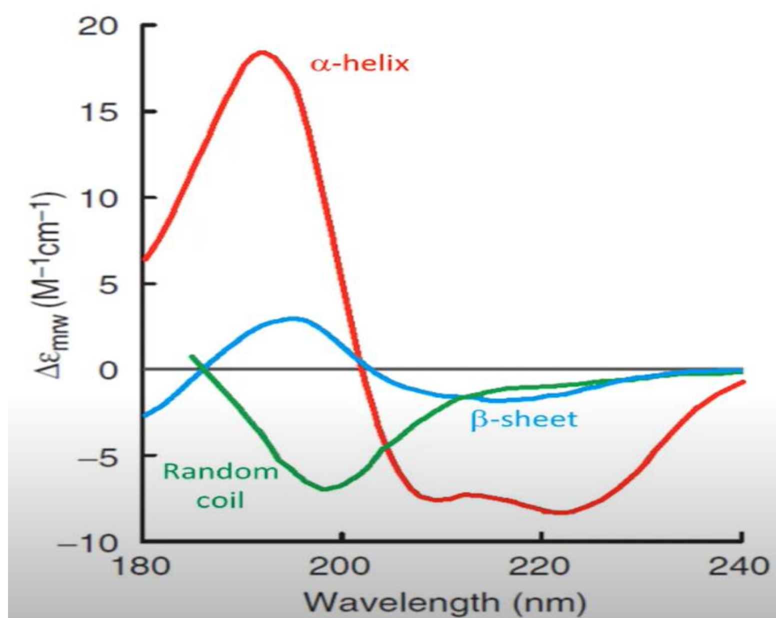


Figure 2 CD result of α-helix, β-sheet and random coil

Nevertheless, CD spectroscopy is very expensive and the operation has a lot of requirements. For instance, cooling down system is required to make the temperature low and stable, and nitrogen must be provided constantly to avoid the formation of ozone. Moreover, the CD result is more complex than the expectation of this project because the information contains not only the folding extent, also the CD result can be different from protein to protein.

1.3.2 Aggregation-induced Emission Fluorescence

Fluorescence is the other method used in this project for measuring protein folding state. When a material is shined by a given wavelength of light, the optically active molecules will absorb the energy of the light and reach the excited electronic state, this process is called excitation. However, the molecules cannot stay in such high energy state for too long, most of excess energy will be emitted immediately by heat, and the remain energy will emit through the form of light, this process is called emission and the emitted light is called fluorescence. Finally, the molecule is back to the original energy state. According to the equation of $E = hv$, the frequency of emission light should be lower than the frequency of excitation light because of the energy loss of heat, therefore, the wavelength of emission light is longer.

Aggregation-induced emission (AIE) concept was brought up in 2001 by Chair Professor

BenZhong Tang and his group in Hong Kong University of Science & Technology. It has drawn more and more attention since it debuts because of its excellent performance and broad prospects. Instead of weakening or quenching of the traditional luminescence in concentrated solution, AIE materials show no emission in dilute solution but intensive luminescence at high concentrations. The mechanism of this phenomenon is suggested in many aspects, including the restriction of intramolecular motion (RIM), inhibition of TICT process, J-aggregates, excimer formation and excited state intramolecular proton transfer (ESIPT). The common AIE materials nowadays include tetraphenylethene (TPE), tetraphenylpyrazine (TPP), 9,10-distrylanthracene (DSA), cyanostilbene, quinoline-malononitrile (QM), silole, and organoboron complexes.

Tetraphenylethene maleimide (TPE-MI) is synthesised by the AIEgens TPE and thiol-reactive group maleimide (MI), result in possessing both AIE phenomenon and reactivity to thiol. According to the research, TPE-MI is non-emissive itself but can produce fluorescence when it is bonding with thiol, the excitation and emission wavelength are 350nm and 470nm respectively, which shows the large Stocks' shift. The hypothesis is that when the protein unfolds from folding, the bio-thiol (Free cysteine) starts to reveal itself, which was buried in the folded protein originally, then the MI group will react with the bio-thiol and bond together, at high concentration, the fluorescence will be detected, which represents the amount of unfolded protein. Comparing to the CD technique, the AIE fluorescence is more affordable and the information it shows is directly related to protein unfolding, which is easier to analyse.

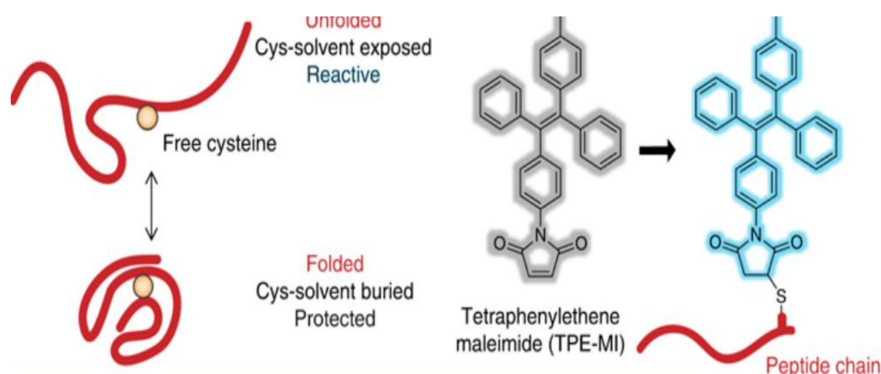


Figure 3 The mechanism of TPE-MI reacts with unfolded protein

Chapter 2 Literature review:

2.1 Aggregation-induced Emission

The concept of AIE was first found from 1-methyl-1,2,3,4,5-pentaphenylsilole by Chair Professor BenZhong Tang and his group in Hong Kong University of Science & Technology in 2001 (Luo et al. 2001). The experiments show that the aggregation greatly boosts emission efficiency of the silole, turning it from a weak luminophore into a strong emitter, unlike the traditional fluorescence dye quenching at high concentration. This phenomenon is then called by them as aggregation-induced emission (AIE), it provides a great possibility and potential on fluorescence measuring.

As years of the development of AIE, many AIE fluorescent sensors have been developed (Gao & Tang, 2017). The subjects to be detected by AIE sensors include ions, small molecules, microenvironment sensing, stimuli response, biological macromolecules, cellular processes and pathogens. Also, the benefit of AIE system is furthermore investigated: such as excellent signal-to-noise ratio, strong photostability, large Stokes' shift and high portability.

A practical example is using AIE to accomplish protein detection and quantitation (Tong et al. 2007). In 2007, Hui and his group developed three derivatives of TPE in order to testify the possibility of protein detection and quantification. The protein used is bovine serum albumin (BSA). Three AIEgens are all non-luminescent in dilute solution but luminescent when aggregated. Finally, after series of testing using NMR spectra, Mass spectra, FL spectra and X-ray, the third derivative is considered the best dye because of its stability, large Stocks shift and high fluorescence intensity, the unknown BSA concentration can be indicated according to the data.

2.2 AIE detecting protein folding state

Maleimide group is well known thiol-reactive, combining with the AIE core tetraphenylethene, it is theoretically reactive with thiol and able to emit fluorescence at high concentrations. The synthesis and research were done in 2010 by Yang and his group (Liu et al.2010). Surprisingly, the experiment shows that TPE-MI and thiol not only have the reactivity, result in the fluorescence at 470nm by giving the excitation at 350nm, also specificity and sensitivity. When

a protein is on folded state, the bio-thiol is buried deeply in the folded protein, so that there is no fluorescence after adding TPE-MI, whereas when the protein is denatured, the secondary structure is destroyed and the thiol is exposed, adding TPE-MI can cause fluorescence. The specificity is proven by testing TPE-MI with other competitive thiol reactant, such as ubiquitin, N-methylmaleimide and glutathione, they all result in no increase in fluorescence. Furthermore, the researchers discovered the reactivity of TPE-MI in real bioenvironment, fluorescence in endoplasmic reticulum (ER) is clearly highest in cells as ER is believed the main area of protein synthesis and folding.

Another derivative of tetraphenylethene is also found able to detect protein folding/unfolding, instead of bonding with thiol in unfolded protein like TPE-MI, it is reactive with folded protein (Hong et al. 2010). This compound is sodium 1,2-bis[4-(3-sulfonatopropoxyl) phenyl]-1,2-diphenylethene (BSPOTPE). Comparing to TPE-MI, the drawbacks of this compound are smaller Stocks' shift and more complicated synthesis.

2.3 Amyloidosis

The polypeptide chain in cells always tend to find the lowest energy structure, so the proteins are mostly folded in human body, in addition, natural selection makes protein folding process more rapid and efficient, however, misfolding can be happening when the cells experiencing mutation (Dobson 2003). The diseases caused by misfolded protein belong to amyloidosis. Nowadays, amyloidosis is always detected by MRI and PET, due to the limitation of instability and non-specificity, testing on body fluid is hardly used in amyloidosis diagnosis.

2.4 Circular dichroism measuring protein structure

Circular dichroism is a property which material performs when it experiencing circularly polarised radiation, shown as the different absorption to the left and right circularly polarised components (Hurlburt 2019). Proteins are usually optically active and chiral, so CD is a useful tool to measure the protein secondary structure. In the paper 'The application of circular dichroism to studies of protein folding and unfolding', the authors illustrate the differences can be obtained when the proteins are folded and unfolded (Kelly & Price 1997). Depending on different types of protein, the change of ellipticity can show on the far-UV wavelength in 200nm – 220nm or near UV wavelength in 250nm-300nm.

For example, β-Lactoglobulin is a typical β-sheet predominate protein, has low ellipticity peak

at the wavelength of roughly 195nm and 216nm comparing to α-helix predominate protein (Matsuura & Manning 1994). Yet the protein secondary structure is a composition of α-helix and β-sheet, so the peak wavelength can vary. The researchers have done the CD measurements on β-Lactoglobulin over a range of pH and temperature, found the conclusion that pH does not affect the secondary structure of β-Lactoglobulin while after heat treatment will result in more β-sheet content.

2.5 Technologies of refolding protein

Formation of inclusion bodies in cells is the result of aggregation of misfolded proteins (Singh et al. 2015). The method nowadays used for refolding protein can be divided by three steps: isolation of inclusion bodies, solubilization of inclusion bodies and refolding, however, the whole process is usually labour intensive, and the yields are often low. The paper 'Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process' talks particularly about the mild solubilization method that can preserve the healthy protein structure while solubilizing, furthermore, getting higher refolding yields. In short, the researchers used relatively lower concentration of chaotropic agents comparing to the traditional way of solubilization, high hydrostatic pressure, high pH buffers and detergents play important role in order to accomplish the goal.

The paper 'Strategies for the recovery of active proteins through refolding of bacterial inclusion body proteins' demonstrates the procedures of inclusion body isolation and solubilization (Vallejo LF & Rinas U 2004), discusses the advantages and disadvantages of well-developed robust refolding techniques such as direct dilution as well as less common methods such as diafiltration and chromatographic. Direct dilution is considered the simplest refolding procedure, whereas it has serious drawbacks which are either huge refolding vessels are required, or the additional cost-intensive concentration steps are needed. On the other hand, dialysis and diafiltration allow the protein solution renaturing gradually, which results in more aggregation comparing to direct dilution, additionally, the non-specific adsorption of protein to the membrane may negatively affect the refolding yields. The paper also discusses the refolding yields change due to altering physical and chemical features, such as low temperature generally can support the productive folding pathway but lower the refolding rate, and the chemical L-arginine is commonly used for enhancing refolding yields.

2.6 Vortex Fluidic Device

Vortex Fluidic Device (VFD) has been proven its value in the areas of biocatalysis, organic synthesis, materials synthesis and probing self-organised system (Britton, Stubbs, Weiss & Raston 2017). In the nanocarbon material field, continuous flow processing are widely used such as VFD, and many advantages of continuous flow processing have been found including decoration nanoparticles on carbon nanomaterials, lateral 'slicing' of carbon nanotubes, exfoliation and scrolling of 2D carbon nanomaterial, synthesis of hybrid carbon nanomaterials involving microorganisms, bottom up fabrication of other carbon nanoforms and self assembly of C_{60} and C_{70} molecules (Vimalanathan & Raston 2017). In the material synthesis and biocatalysis areas, Luo and her group conducted the experiment of using VFD to synthesis macroporous bovine serum albumin-based microspheres (Luo et al. 2018). Finally, the authors conclude that using VFD thin film microfluidic processing platform to synthesis and cross-link BSA nanoparticles is a relatively new method, but it is proven that under confined mode of operation, the process is effective and optimized.

Shear stress in micrometer-wide also has been proven rapid and effective (Yuan et al. 2015). The traditional method of refolding proteins such as dialysis can take over one night, but the shear stress-mediated refolding method can finish within few minutes. By calculating the shear forces, the authors state the fluid behaviour can be estimated, therefore it is easier to control the whole process. An boiled egg is essentially made out of denatured protein, so as the further research of the rewarded Ig Nobel prize: A chemical recipe to partially unboil an egg, a collaborate research was conducted by Flinders University and California University in 2017 based on using VFD to help protein folding (Britton 2017). The experiment generated a fluid behaviour model, the shear stress is therefore estimated, and the Faraday waves are monitored, finally, the results shown on the HEWL activity assay kit and CD spectrophotometer are promising.

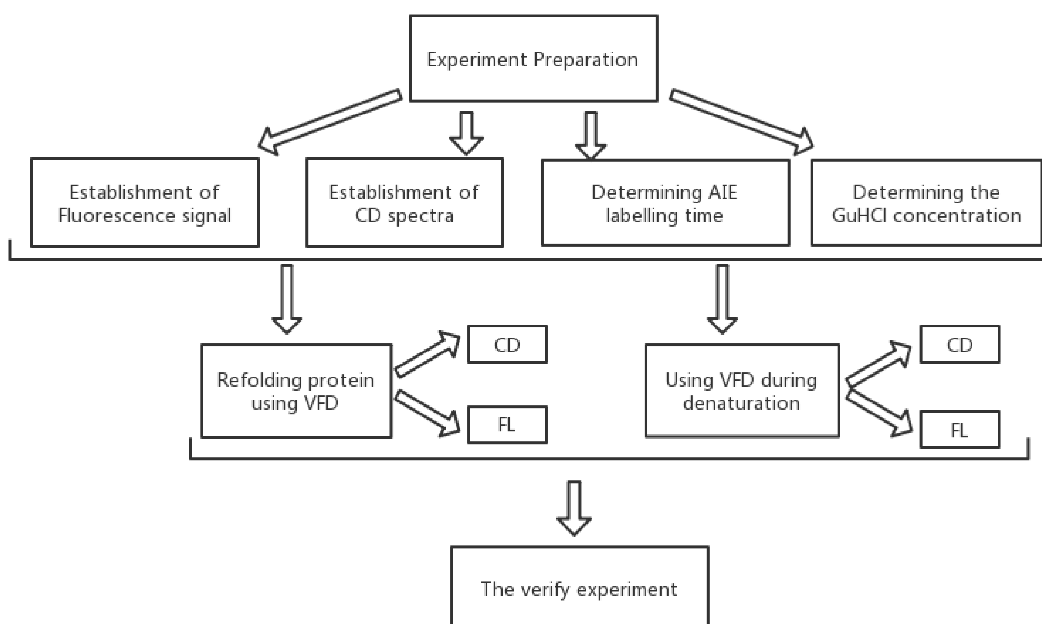
Chapter 3 Materials:

The materials used in this project include the protein β-lactoglobulin, denaturant Guanidine hydrochloride and PBS buffer, they are all purchased from Sigma-Aldrich. The AIE flurescence dye TPE-MI is kindly provided by Latrobe University, Department of Health and Engineering.

The VFD used in this project is from Flinders University College of Science and Engineering.

The fluorescence is measured by a Cary Eclipse Fluorescence Spectrophotometer, and Circular dichroism is recorded by a Jasco CD spectrometer.

Chapter 4 Method and Result:



Flowchart 1 - Methodology

4.1 Experiment preparation

The solid AIE fluorescence dye TPE-MI is synthesized by Latrobe University then it is made to stock solution at the concentration of 50 μM. The procedure is firstly dissolving it in small amount of DMSO until its fully dissolved then adding PBS buffer into the solution, after that, the stock solution is saved in refrigerator at 4 degrees for usage. In each time of experiment, 10ml of TPE-MI stock solution is extracted and placed under room temperature for 1 hour before using.

Protein β-lactoglobulin is powder-like and needed to be stored in refrigerator at 4℃. In each

time of experiment, weighting proper amount of β-lactoglobulin and making it to 50 μM solution, where only PBS is the solvent, after making the solution, placing until its temperature is room temperature. The denaturant GuHCl is 6M concentrated liquid when purchasing, store requirements are room temperature and shade. The denaturation process is conducted by mixing the protein solution and the denaturant solution, the concentration and ratio will be investigated at the following steps.

Fluorescence spectrophotometer needs to be turned on before 15 minutes of using, excitation wavelength and emission wavelength is 350nm and 470nm respectively, because the emission intensity is the desired value, so the spectrophotometer is set to emission mode and the emission wavelength is 400nm to 600nm, the sample volume within the cuvette is 3ml. CD spectrophotometer needs to be flushed with nitrogen at least 5 minutes before using, also nitrogen flow rate requirement has to be followed strictly for different range of wavelength. The sample volume within the cuvette is 250μL. There is no preheat requirement for VFD, but in order to maximize the shear stress and Faraday wave, it is usually 2ml liquid in a 20 mm VFD tube, the parameters of VFD for this project are 5000rpm rotational speed, 45 °tilt angle, CW and 10 minutes operating.

4.2 Establishment of fluorescence signal

The AIE dye TPE-MI is designed to react with bio-thiol, which will be exposed when the protein is on unfolding state, then emit fluorescence signal at 470nm by giving the excitation at 350nm, in addition, the TPE-MI itself is non-luminance. The first part of the experiment is to identify the fluorescence. For the experiments containing denatured protein and TPE-MI, the denaturant GuHCl and TPE-MI were added at the same time so that the AIE labelling time can be a constant.

The 'Prescan' function of fluorescence spectrophotometer can effectively determine the excitation and emission wavelength of the fluorophore. According to the Prescan result, excitation is on the wavelength of 350nm and emission is on the wavelength of 470nm, which matches with the previous study.

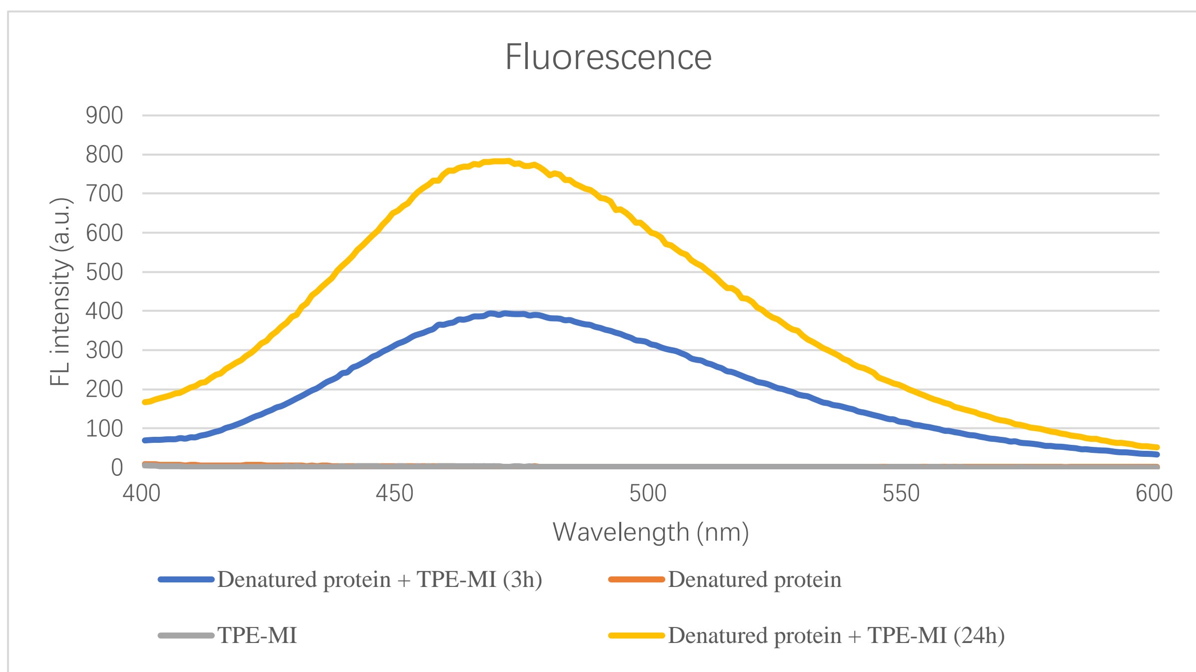


Figure 4 Fluorescence of denatured protein, fluorescence of TPE-MI, fluorescence of 3h denatured protein + TPE-MI and fluorescence of 24h denatured protein + TPE-MI

The figure shows the background fluorescence, which is the fluorescence caused by each of single reactant in the experiment, denatured protein and TPE-MI, having no signal on the emission wavelength. On the other hand, the distinct fluorescence peak at 470nm of blue line and yellow line showing emission due to TPE-MI labelling denatured protein. In addition, the fluorescence of 24 hours reaction is clearly bigger than 3 hours reaction, which means the fluorescence intensity will increase as the denaturation process going.

One thing needs to be emphasized is that given on the properties of TPE-MI, it can bond with the free-thiol which is exposed when the protein is unfolded, and emit fluorescence when experiencing excitation, the fluorescence will not be eliminated once it bonded with the free-thiol, whether the protein is back to folding state or not. Therefore, it is necessary that adding TPE-MI after performing VFD in refolding experiment, in addition, in order to control the variables, the AIE dye TPE-MI was always added after the denaturation time in the fluorescence measurements conducted in this project even it is not essential in the unfolding experiment.

4.3 Establishment of CD spectrum

Unlike the AIE fluorescence method, the circular dichroism is one of the intrinsic properties of a protein, therefore TPE-MI is not needed in this step. Furthermore, instead of having a peak intensity on a particular wavelength as fluorescence, the method circular dichroism usually not just focuses on one wavelength but the wide range from 180nm to 290nm, for example, a pure α-helix protein usually have a large positive ellipticity peak at 195nm, two negative peaks on the wavelength of 210nm and 225nm and a distinct gap on the wavelength of 215nm. The different value on the different wavelength usually contains more information than people could be aware since protein secondary structure is very complex.

The protein used in this experiment, β-lactoglobulin is a typical β-sheet predominant protein, it will have a big negative peak on the wavelength of 216nm, which represents the amount of β-sheet content according to literatures. Additionally, due to the limitation of the machine and PBS buffer, a lot of noise exists on the wavelength between 180nm and 200nm, so the experiment ignores the result before 200nm and only focuses on the wavelength of 216nm.

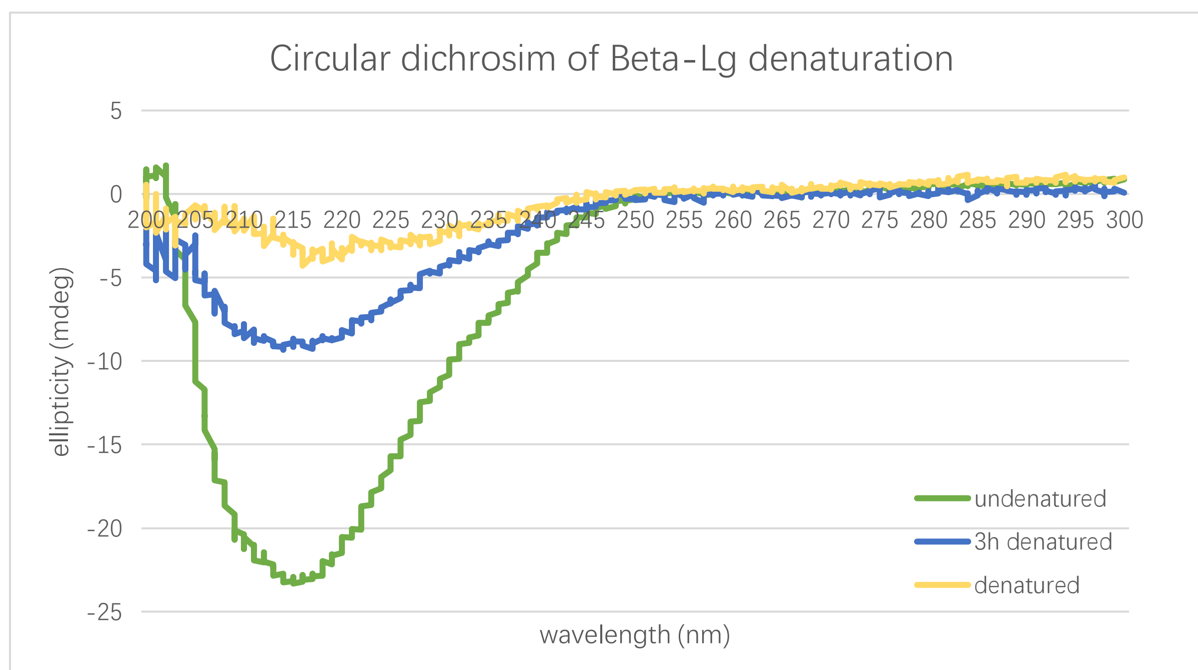


Figure 5 The CD results of undenatured protein, 3h denatured protein and 24h denatured protein

The figure shows the different ellipticity of the protein under 3 different denaturation states,

analyzing the figure, it is clearly that the protein will have large negative peak on wavelength at 216nm when the protein secondary structure is more complete, and more β-sheet content. After 24 hours denaturation, the ellipticity of the protein at 216nm is lowest at negative 3 millidegrees. However, it is the opposite result to the study conducted in 1994 by James and Mark, where they performed heat treatment to β-lactoglobulin (60min 90 °C), the study showed increased Mean Residue Ellipticity at the wavelength of 216nm after heat treatment under pH 7, they stated it is consistent with a large increase in β-sheet content. The unit Mean Residue Ellipticity is theoretically proportional to the Ellipticity so it can not cause opposite result, therefore, the author thinks that the two denaturation method, high concentration of chaotropic agent and heat shock, can cause different result to β-sheet content even they are both denaturing.

At the stage, the analysis methods of fluorescence and CD are established, the different β-sheet content of different β-lactoglobulin solution can be compared given the result of the measurements.

4.4 AIE labelling time

At previous steps, the AIE dye TPE-MI was added into the solution at the same time as the denaturant, since in that case, it can be make sure that every unfolded protein is detected and labelled by TPE-MI, therefore the fluorescence intensity is directly determined by the number of unfolded protein. However, once the dye is bonded to the free thiol, it will generate fluorescence even if the unfolded protein back to folding state, therefore, it is meaningless to add TPE-MI with denaturant at the same time when doing refolding experiment. Hence, knowing how long the TPE-MI can finish labelling unfolded protein becomes crucial to this project.

Control experiment requires only one variable at a time, in this case, the one variable is the AIE labelling time, so the amount of unfolded protein has to be certain. Low concentration of denaturant (2M) and long denaturation time (over 24 hours) is used in this step to make sure there is no more unfolded protein are produced during the labelling process.

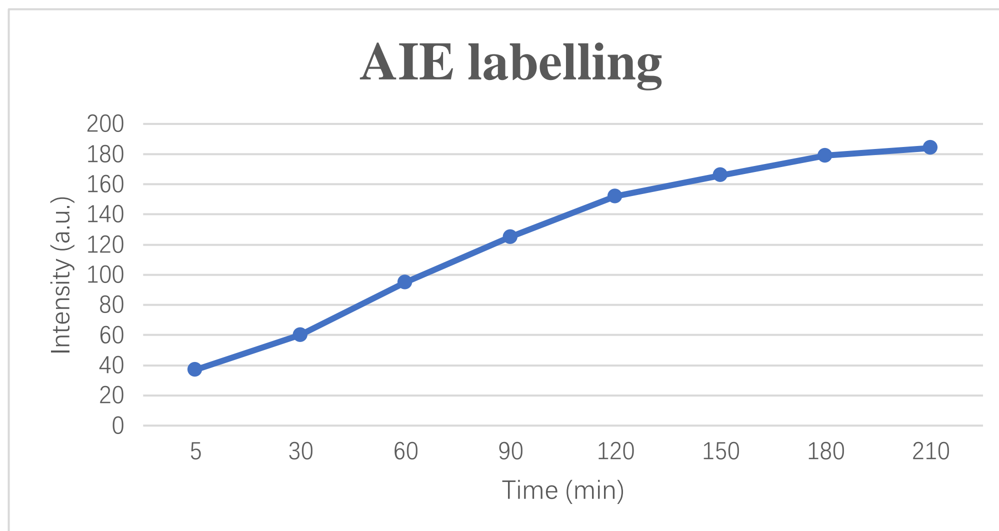


Figure 6 The fluorescence intensity change during AIE labelling

Figure 6 shows the fluorescence intensity change during 210 minutes of AIE labelling after long denaturation time. Since the low denaturant concentration is used, the intensity is low in general. The intensity increases slowly in the first two and a half hours then tends to be stable after 3 hours, even though it still has an increasing trend and hard to see the maximum point.

4.5 Determining the concentration of GuHCl

For the experiments that will be conducted afterwards, one is refolding protein using VFD, which requires the denaturation process is finished and no more denatured protein will be further produced. The other one is using VFD during denaturation, which requires denaturation is still going and part of proteins are already denatured.

Since the concentration of protein solution is already certain, there are two variables can affect denaturation, denaturation time and denaturant concentration. In this step, denaturation time of 3 hours and 24 hours are chosen, for the 3 hours denaturation, it is expected to be used on the unfolding experiment, which the reaction is still going and some of the proteins are denatured. For the 24 hours denaturation, it is expected that the reaction is finished and the number of denatured protein hardly increases, therefore it is suitable for the refolding experiment. To find the optimal concentration for the two timing, the fluorescence intensity of using different denaturant concentration over 24 hours is compared. Since the original concentration of GuHCl solution is 6M when purchasing, the concentration of denaturant in this step is from 6M to 1M

as the gradient is 1M.

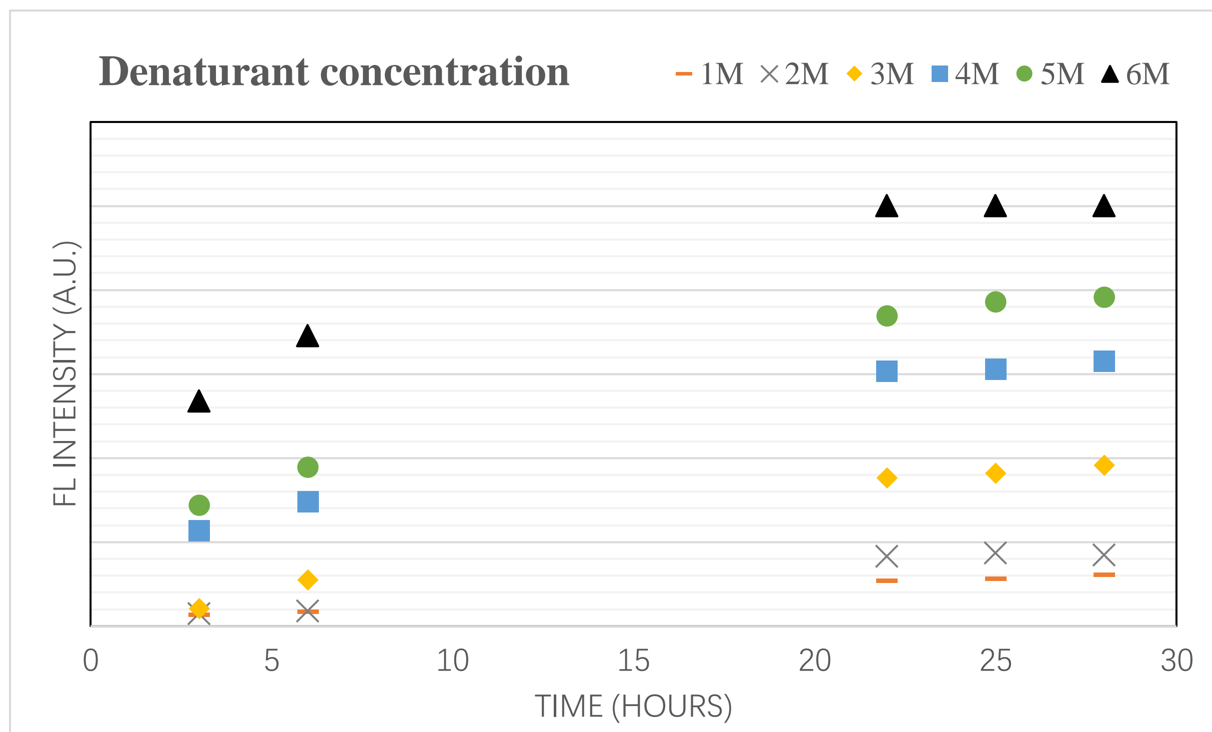


Figure 7 Fluorescence intensity of using different concentration of GuHCl

From figure 7, it can be seen that after 24 hours denaturation, the fluorescence intensities of different concentration all tend to be stable even there is still slightly increment over 5 hours. The intensity of concentration 6M is unchanged because it was overrange of the fluorescence spectrophotometer, therefore, it can be assumed the reaction is nearly finished after 24 hours. In addition, the fact that higher concentration can cause higher intensity after 24 hours denaturation suggests that in each point, there is still undenatured protein, and the reason why the reaction stops is the loss of effect of denaturant.

For the three hours denaturation, the half amount of the total intensity indicates that the reaction is halfway through, and the increasing intensity shows the denaturation is still in progress, which match the requirements of the second experiment. Finally, the denaturant concentration of 4M is chosen in this project.

4.6 Refolding protein using VFD

4.6.1 CD measurement

After the preparation of the experiment and establishment of the experiment conditions, this stage is to investigate the protein refolding process using VFD, where fluorescence and CD are the two measuring methods.

Comparing to the other traditional methods of refolding protein, VFD has the advantage of easy to control, which can be accomplished by adjusting the parameters including rotation speed, rotation direction and tilt angle. In this project, rotation speed 5000rpm is based on the previous study, 45 ° is the optimal angle for the dynamic fluid according to the researches. Temperature can be another affecting factor in this experiment because the high rotational speed will obviously create heat and furthermore, increase the temperature, however, study shows for a liquid whose principle component is PBS buffer, the maximum temperature change is 2 degrees, which can hardly influence the protein structure.

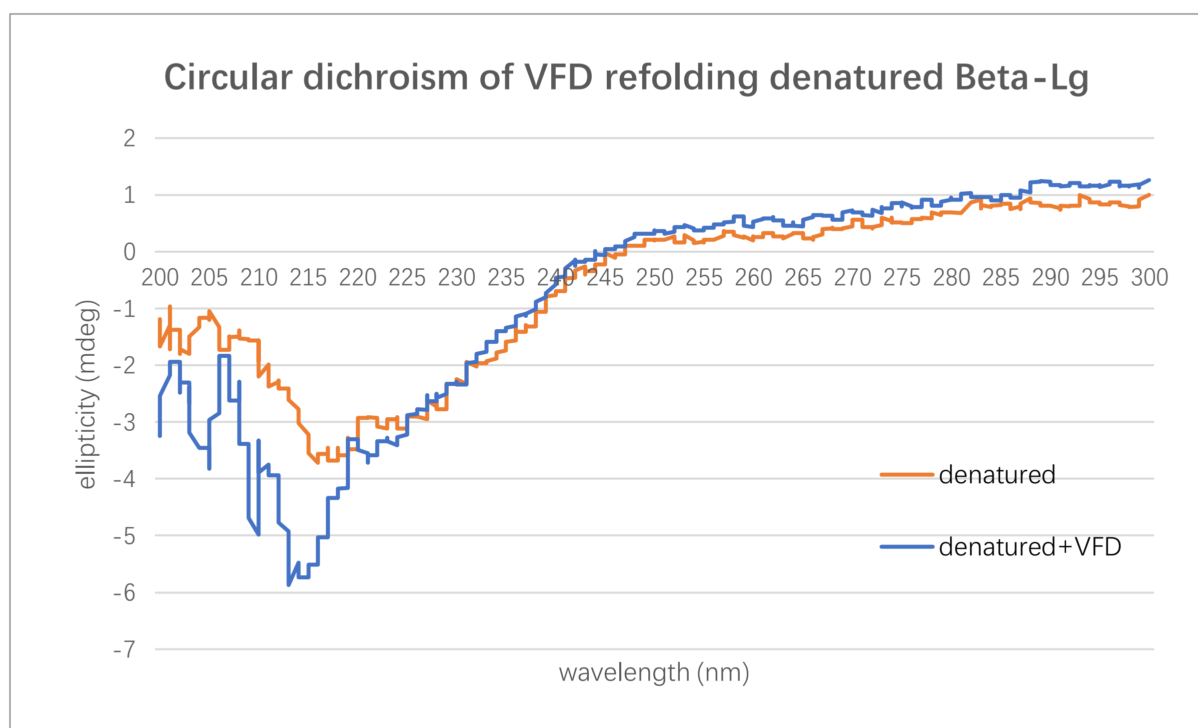


Figure 8 The CD result of after 24h denatured protein and after 24h denatured protein + VFD

When analysing the CD data, a lot of noises can be obtained from the line graph drawn by the raw data, which means the ellipticity lines are fluctuant and unstable even though the main difference at 216nm is shown. The noise indicates that when the protein solution underwent denaturation, the structure of proteins will be more complicated, as well as the number and orientation of optical chiral molecules. Moreover, the high dynamic thin fluid film and high shear stress will even make the inside mechanism more complex. The way of the author has done for signal processing is to find the median value of every 5 consecutive values, then draw the figure using the set of median values, it is a common way of eliminating fluctuating noise interference without distorting the signal. However, figure 5 was drawn from the raw data but few noises were found, it is presumably caused by the larger scale of y axis.

As the previous CD spectrum indicated, for the protein β-lactoglobulin, as the denaturation induced by GuHCl going, the lower ellipticity (Absolute value) at the wavelength of 216nm means the stronger denaturation extent, in another word, less β-sheet content. Figure 8 shows the CD results of β-lactoglobulin after 24 hours denaturation, and β-lactoglobulin after 24 hours denaturation combining 10 minutes VFD performing. It is clearly that the ellipticity increased more than 2 millidegrees (Absolute value) after VFD using, comparing to the less than 4 millidegrees of the total ellipticity of the denatured protein, the increment as the result of refolding is significant.

This protein refolding phenomenon was already observed by the previous study as the literature review mentioned. The following steps are the innovation points of this project, detection method of AIE fluorescence and using VFD during denaturation to make the reaction go backward.

4.6.2 FL measurement

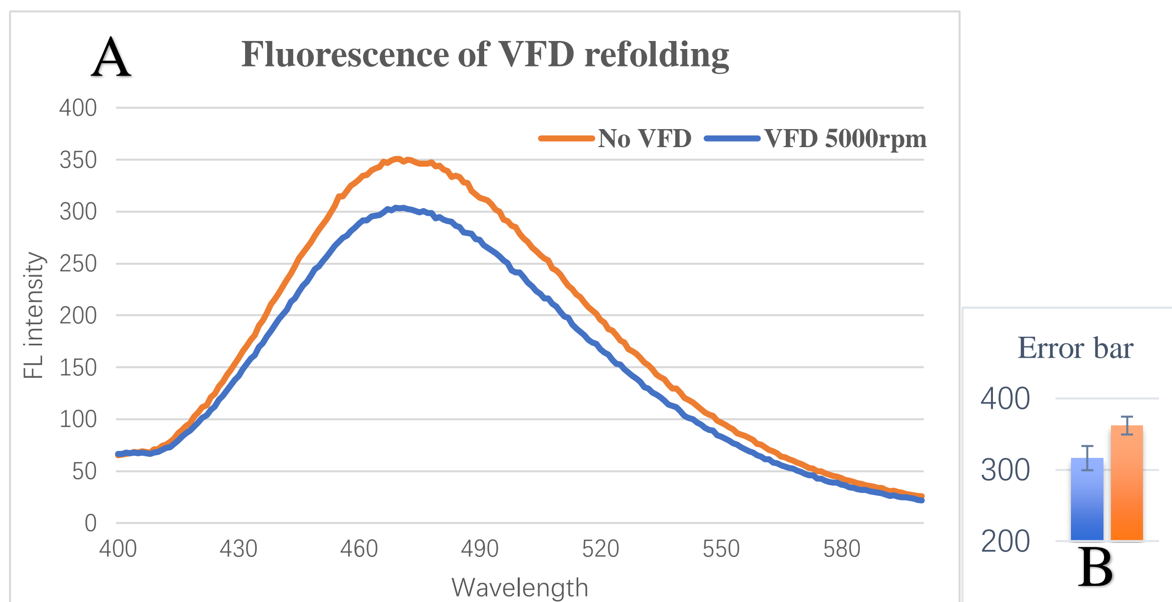


Figure 9 A. The fluorescence result of after 24h denatured protein and after 24h denatured protein + VFD. B. The error bar of experiment 9

Appling the same conditions for fluorescence measuring, before measuring, adding TPE-MI and waiting for two and a half hours after denaturation, the result shown as figure 9 A. The emission wavelength of TPE-MI labeled protein is at 470nm as reported by the reference, as well as the solution after using VFD. The unit of the fluorescence spectrophotometer is a.u. (arbitrary unit), it does not have the exact meaning by it self, however, the different intensity on the same graph can be compared so that the change can be obtained proportionally. The fluorescence intensity of 24 hours denatured protein with TPE-MI after 2.5 hours reaction is around 350, this can be regarded as a known value because the conditions to get this value in each experiment are the same, in order to testify by running multiple experimental data, an error bar (Figure 9 B) is made. The decrement of fluorescence intensity due to VFD performing is relatively stable as around 50, reduced 14 percent of the total intensity, which can be regarded as the refolding efficiency.

At this stage, given on the CD measuring result and fluorescence measuring result, it is confident to say that VFD can effectively refold proteins, making the denatured proteins obtain more complete structure. By performing a dynamic thin fluid film, unlike the traditional ways aiming to denaturant removal such as direct dilute and dialysis, using high shear stress to

renature protein is a novel method.

4.7 Using VFD during denaturation

4.7.1 CD measurement

Although it was already identified in the previous study of VFD refolding proteins, no research has done VFD during the denaturation process, the author thinks it may provide a new idea for amyloid research if using VFD can stop or even reverse protein denaturation. In this experiment, the researcher chose 3 hours as mentioned previously, which is believed consistent with half of denaturation process and healthy proteins, denaturant, and unfolded proteins are all present in the solution.

In order to control the variables, the two measurements are done at the same time, which means while one of the 3 hours denatured protein solution was standing, the other one was performing 10 minutes VFD. Other conditions such as rotational speed, tilt angle and solution concentrations are all the same as the previous step, so that the only variable in this experiment is the VFD performing time.

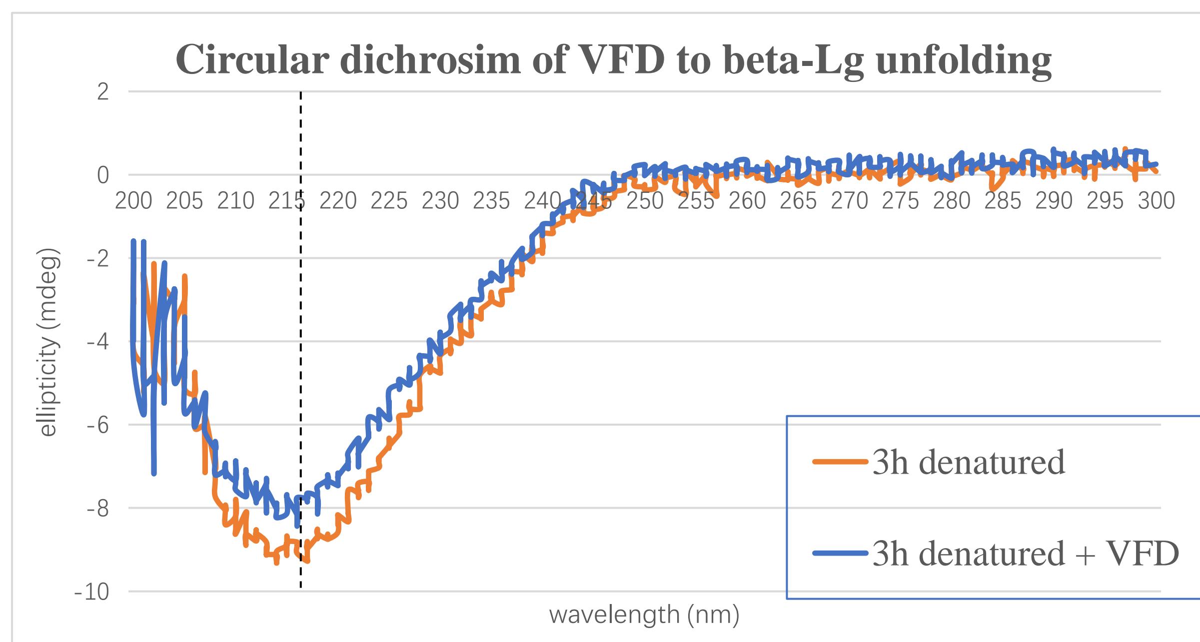


Figure 10 The CD result of after 3h denatured protein and after 3h denatured protein + VFD

Unfortunately, instead of reversing the denaturation, using VFD even promoted the process, resulting in lower ellipticity (Negative value) at the wavelength of 216nm and more denatured proteins, although the change is small (The raw data was also processed as the procedure of 4.6.1).

4.7.2 FL measurement

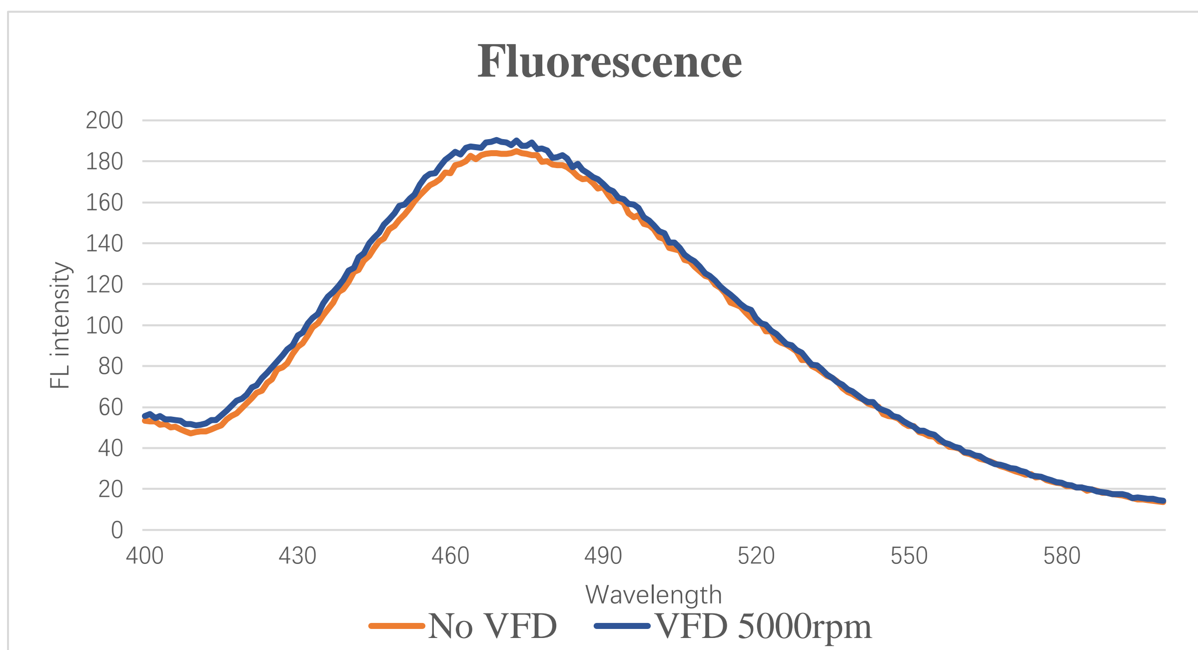


Figure 11 The fluorescence result of after 3h denatured protein and after 24h denatured protein + VFD

The fluorescence intensity of two solutions at the wavelength of 470nm shows the same result: using VFD during denaturation process (high concentration of chaotropic agent is still present in the solution) results further but small amount of denaturation.

Considering the mechanism of VFD is to create a dynamic thin fluid film by applying high rotational speed, it will certainly act like a mechanical catalyzer and promote the reactions. In this case, high concentration of chaotropic agent GuHCl is still present after 3 hours denaturation, so that the reaction of GuHCl and undenatured protein is promoted because of the high shear stress and more sufficient mixture. Additionally, given on the result found on Figure 8 and Figure 9, when all the conditions are satisfied, it cannot be neglected that the VFD is refolding the denatured protein at the same time, which means during this time, there are two processes in the solution in this experiment due to applying VFD. One is VFD refolding

proteins and the another one is VFD boosting the denaturation process. Moreover, the lower ellipticity and higher fluorescence intensity suggests that the mechanism of boosting is dominating.

4.8 The verify experiment

Combining the findings on the previous two steps, the main purpose of this project: protein structure manipulation is accomplished by using VFD on the certain conditions, which can be concluded that: first, VFD can turn unfolded protein back to folded protein, second, when the proteins are undergoing denaturation process, VFD can promote the reaction of proteins and denaturant and result more denatured proteins.

However, the two experiments were done and identified separately, depending whether the concentrated denaturant is present in the solution, experiment 1 was done after 24 hours denaturation, which is considered complete reaction, and experiment 2 was done after 3 hours denaturation, which is believed during the reaction. Since the only denaturation method used in this project is high concentration of chaotropic agent GuHCl, the conclusion does not stand for other approaches, results may vary from the methods of heat shock or urea.

A combination experiment was conducted to further identify the findings. The way of doing that is firstly repeat the experiment 1, which is measuring after 24 hours denaturation, then simulate the experiment 2 by adding more denaturant into the solution rather than using 3 hours denaturation directly, and measuring again. Therefore, the changes of the whole process can be obtained and compared by one experiment.

Since there are several limitations of CD machine such as long testing time (15 minutes per sample) and strict requirement of protein concentration (depends on the machine), as well as the more complicated and less visualized data comparing to AIE fluorescence, this experiment chose only fluorescence as measuring method.

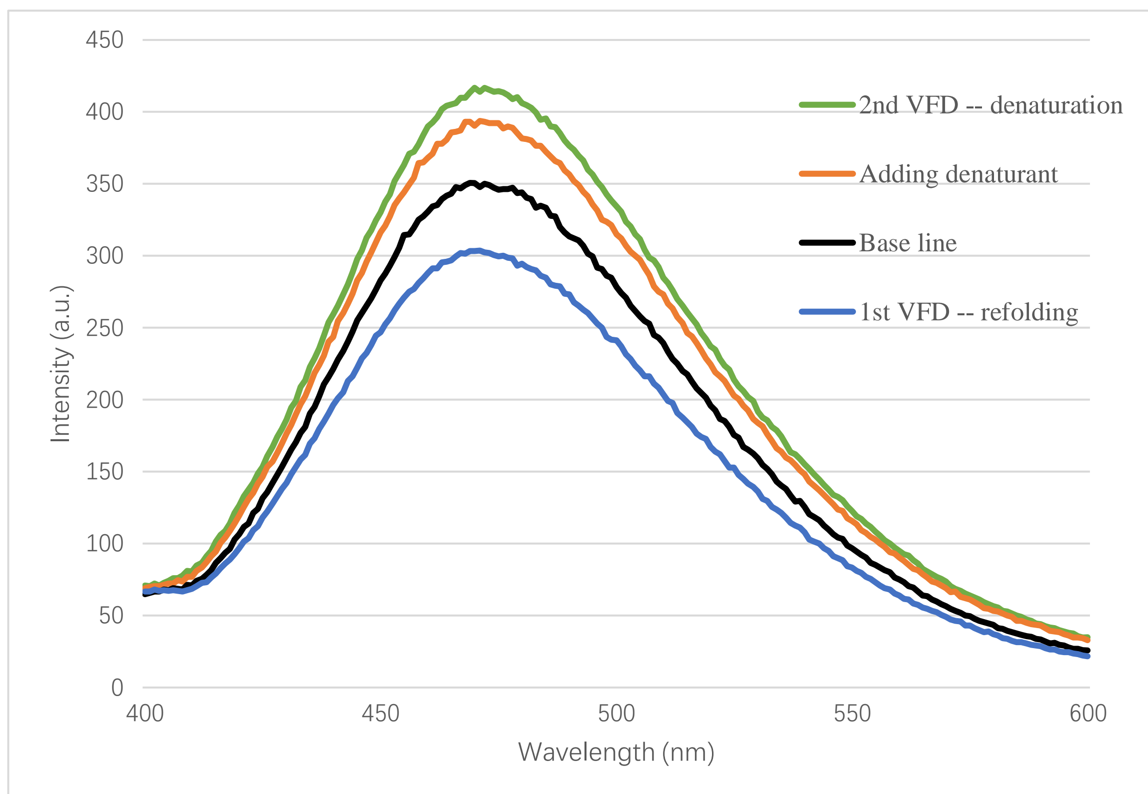


Figure 12 The verify experiment. Four fluorescence testings are in order: after 24 hours denaturation; after 24 hours denaturation and VFD; adding more denaturant after VFD; performing VFD again(Fluorescence)

As shown on figure 12, the black line is the emission fluorescence intensity of the protein solution after 24 hours denaturation and 2.5 hours labeling by AIE dye TPE-MI from wavelength 400nm to 600nm, similarly like the blue line on the figure 9A. Because the other 3 steps of this experiment represented by the other three lines are all based on this solution, the black line is therefore called base line.

The blue line represents the fluorescence intensity of the base solution (Base line) after applying first time of VFD, this scenario is to simulate the experiment 1. As expected, the fluorescence intensity at 470nm dropped from 350 to 300 because apparently VFD refolded part of the unfolded proteins and result less free-thiol can be bonded with TPE-MI, the refolding proportion is about 14%. As mentioned previously, even the unfolded proteins refold will create less exposed free-cysteine to react with AIE dye, if TPE-MI were bonded with the unfolded protein earlier, refolding will not decrease the fluorescence intensity. Therefore, the first time VFD was performed before adding TPE-MI to the base solution (Black line).

Then more denaturant was added into the solution, the aim of this step is to simulate the part 4.7.2. The proteins to be denatured can be divided by two portions: the proteins just refolded due to VFD and the undenatured proteins survived from the denaturation. Because of the greatly reduced undenatured protein concentration comparing to the part 4.7.2, low concentration of denaturant (few drops of 1M concentration) and short time period (30 minutes) is used in this step in case of the fluorescence over range because of the fast response.

The last step of the combination experiment is to perform VFD again after adding denaturant. Unlike the first time VFD making the fluorescence intensity deceased, this time VFD makes fluorescence intensity increased slightly, roughly 6 percent of the total intensity, which is consistent with the result obtained from experiment 2. Assuming the refolding process is still working in this step and resulting 14% refolded proteins from the total unfolded proteins, VFD promoted the denaturation process about 20% , in the other word, even VFD is refolding proteins at this step, but still more denatured proteins are produced because the high rotational speed of VFD increased the denaturation efficiency.

4.9 Analyzing the CD result from DichroWeb

The circular dichroism property of a protein is very complex, the analysis usually involves multiple band of wavelength in order to determine the composition and structure of the protein. In this project, since the protein β-lactoglobulin has its own characteristic peak at wavelength of 216nm which is reported by the previous studies, also due to the limitation of the CD machine, loud noise presented at the wavelength lower than 200nm and higher than 300nm, the analysis of circular dichroism in this project mainly focus on the changes at wavelength 216nm. Nevertheless, there are still some information from the CD figures is missed. Therefore, the author brings in another analysis method to this project as a reference which is the website called DichroWeb.

Figure 13 DichroWeb home page

DichroWeb is for online analysis of protein Circular Dichroism spectra. It is established in 2001 and hosted by the Department of Crystallography at Birkbeck College, nowadays has been used by thousands of scientists in over 50 countries to gain structural information about their protein samples (Whitmore L. & Wallace B.A. 2008).

The way of DichroWeb works is that it contains massive reference data sets, by matching the result people obtained with the data sets, the system can find the 10 best matches for the protein sample, which also requires the user providing the information related to the measurement such as the protein name, the input units, the wavelength range and the wavelength step. Additionally, a few algorithms can be chosen when analyzing, including Selcon3, Continll, Verslc&Cdsstr and K2D, using different algorithms will give accurate result depending different situation. For example, this project aims to determine the protein β-lactoglobulin denaturation state, so that the algorithm SELCON3 is used.

A

NRMSD:0.603

Helix segments per 100 residues: 1.256

Ave helix length per segment: 13.199

Strand segments per 100 residues: 1.488

Ave strand length per segment: 2.431

Result	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
1	0.118	0.058	0.000	0.044	0.000	0.780	1

B

NRMSD:0.574

Helix segments per 100 residues: 2.050

Ave helix length per segment: 4.000

Strand segments per 100 residues: 1.200

Ave strand length per segment: 7.167

Result	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
1	0.053	0.082	0.144	0.081	0.103	0.537	1

Figure 14 The analysis of Figure 9A obtained from DichroWeb. A. The protein solution after 24 hours denaturation without using VFD. B. The protein solution after 24 hours denaturation combining VFD

NRMSD:0.495

A **Helix segments per 100 residues: 2.352**

Ave helix length per segment: 10.533

Strand segments per 100 residues: 3.062

Ave strand length per segment: 2.392

Result	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
1	0.114	0.132	0.000	0.117	0.244	0.393	1

NRMSD:0.419

B **Helix segments per 100 residues: 5.833**

Ave helix length per segment: 8.383

Strand segments per 100 residues: 4.567

Ave strand length per segment: 4.985

Result	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
1	0.356	0.000	0.000	0.154	0.000	0.490	1

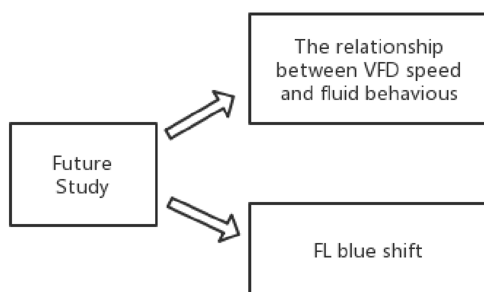
Figure 15 The analysis of Figure 11 obtained from DichroWeb. A. The protein solution after 3 hours denaturation without using VFD. B. The protein solution after 3 hours denaturation and VFD

The parameter NRMSD in the above two figures is the abbreviation of normalized standard deviation (Mao D., Wachter, E. & Wallace B.A. 1982), which represents the accuracy of the analyzing result. All the four NRMSDs are larger than 0.4, which is considered big error, the author thinks that it is because the protein β-lactoglobulin is not commonly used in the previous studies, so there is not many data to match, also the raw CD data in this project has a lot of noise, consequently, making the analysis more difficult. Since the project's aim is to determine the folding state of the protein sample, the values of segments per residues and average length per segment will not be discussed, the report is only focusing on the value of unordered (Disordered).

Figure 14 is the DichroWeb analysis result of Figure 8 in part 4.6.1 (refolding experiment), A is the experiment without using VFD and B is after using VFD, it can be seen that the value of unordered on B is clearly smaller than A, the decrease is about 0.3 of the total, which is consistent with the analysis of Figure 8. Figure 15 represents the analysis result of Figure 10 in 4.7.1 (unfolding experiment), CD spectra of using VFD during denaturation process, the

unordered values show slightly increase after using VFD (Figure 15 B), which is similar with the previous analysis.

Chapter 5 Future work



Flowchart 2 Future work

5.1 VFD rotational speed

Rotational speed is a critical parameter when using VFD, it will greatly affect the dynamic of fluid flow, and furthermore affect the reaction within the VFD tube. The influence mainly depends on the types of fluid. For example, according to researches, when there are two different liquids within the VFD tube, and the majority component is water, the fluid behaviour will change from the rotational speed 3000rpm to 5000rpm. Rotational speed 3000rpm will result the water behave like a single helix, which the two types of fluid are rotating along the same path, theoretically less mix efficiency and lower reaction efficiency Water under 4000rpm usually have irregular behaviour, which is unsure about the influence to the mixture or reaction. When using 5000rpm, the fluid inside the VFD tube tends to be like double helix shape, creating inner shear stress and increasing the mix and reaction efficiency. The rotational speed higher than 5000rpm is usually also the research emphasis because it will certainly create more complex fluid behaviour.

This project also conducted the research of VFD refolding and unfolding proteins under different rotational speed and the results show on the figures below. Because of the small scale

and non-specificity, this experiment used AIE fluorescence as measurement only.

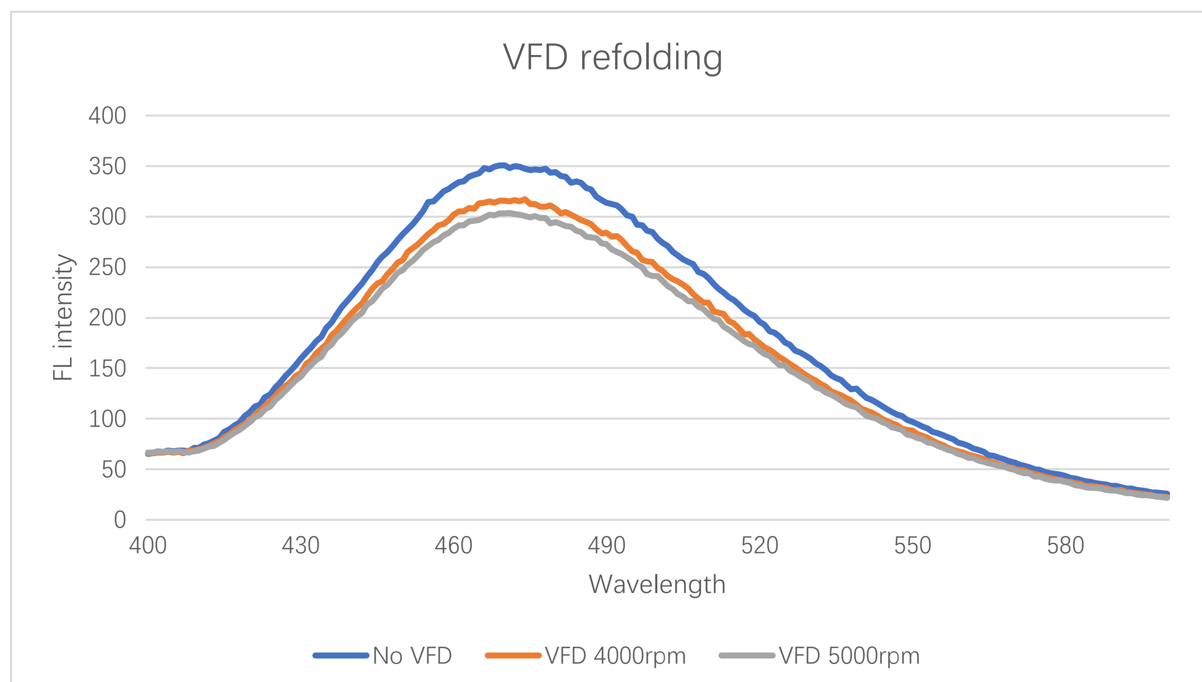


Figure 16 The fluorescence of VFD rotational speed 4000rpm and 5000rpm in refolding experiment

Since the previous study of VFD refolding proteins (Britton J et al., 2017) used rotational speed 5000rpm to refold proteins, in this experiment, the author continued with this option and compared with the fluorescence of using 4000rpm rotational speed to see the difference. As the figure shows, both rotational speeds 4000rpm and 5000rpm caused the fluorescence intensity drops, but the decrease due to 5000rpm is slightly bigger than the decrease using 4000rpm, even though the intensity difference is tiny about 5 machine units. The author supposes that the reason why the previous study used 5000rpm is that 5000rpm is the optimal rotational speed which can make the refolding result maximum, the other rotational speed was not mentioned is because the results were very close.

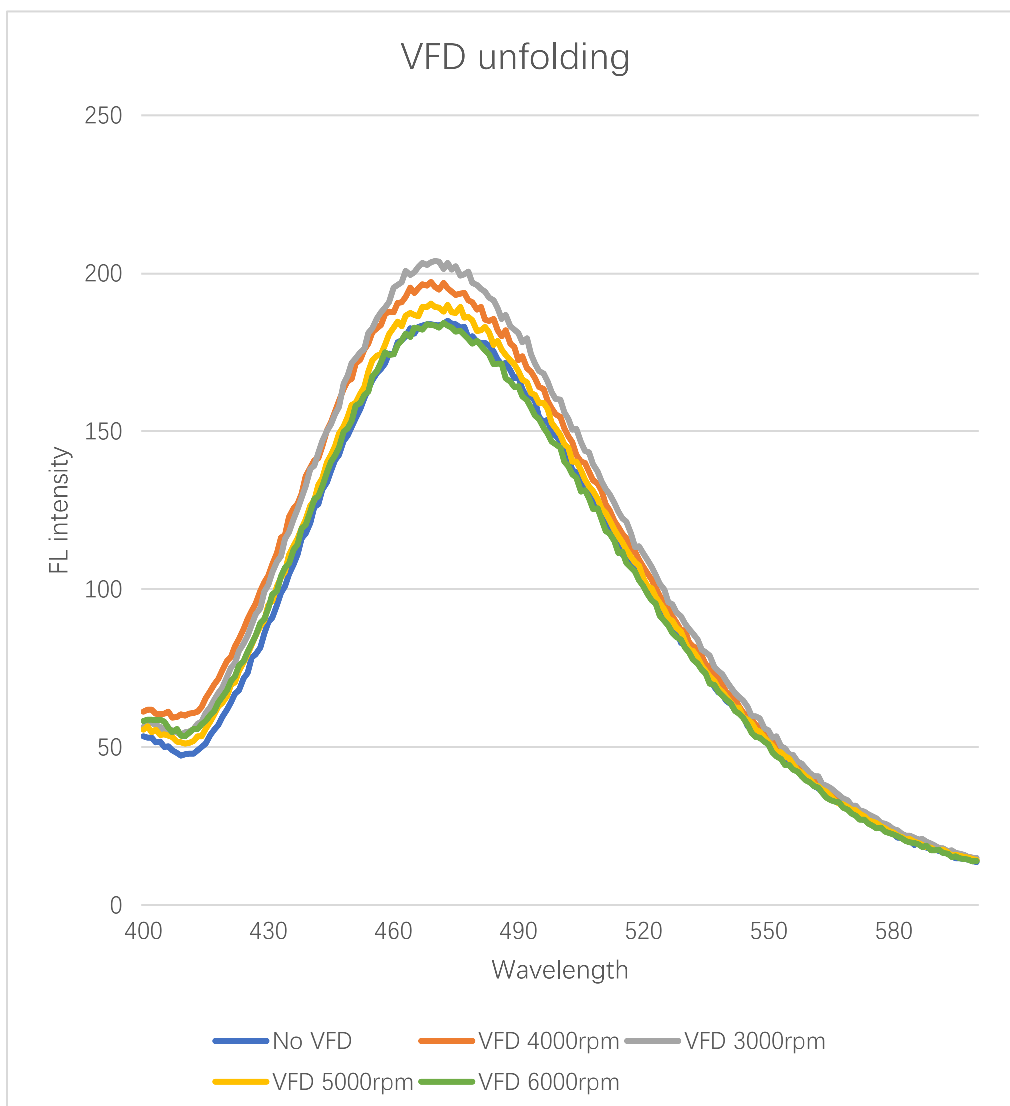


Figure 17 The fluorescence of VFD rotational speed 3000rpm, 4000rpm, 5000rpm and 6000rpm in unfolding experiment

Figure 17 shows the fluorescence of using VFD rotational speed 3000rpm, 4000rpm, 5000rpm and 6000rpm during denaturation process. Since there is no research has been conducted in this area, the author decided to compare the rotational speed from 3000rpm to 6000rpm. It is found that the maximum peak intensity of this experiment is around 200 which is much lower than figure 16 (24 hours denaturation), because of the denaturation time difference. It is also obtained that using VFD during denaturation process can make the fluorescence intensity increases, in other words, promoting the reaction, which is the same result as Figure 11. Interestingly, the increment of fluorescence intensity at 470nm decreases as the rotational speed increases, even though just in a very small amount. Which means that relatively lower

rotational speed can make the denaturation promoted larger, since the rotational speeds 1000rpm and 2000rpm are not used in this experiment, this assumption only suit for 3000rpm to 6000rpm. In the author's explanation, combining the result obtained from Figure 16, higher rotational speed results more refolded proteins and lower fluorescence intensity, it makes sense that in Figure 17 the lower rotational speed has higher intensity, which also indicates that the rotational speed is less affective to the process of increasing denaturation speed then refolding protein.

However, to relate the result obtained from Figure 16 and Figure 17 with the fluid behaviors change due to different rotational speed, more measures needs to be done and fluid dynamic microscopy needs to be taken, it will not be discussed in this thesis.

5.2 Blue shift of FL intensity peak

At the experiment preparation stage, both AIE dye TPE-MI and protein β-lactoglobulin were originally made to stock solution, so that the dissolving time can be saved in each experiment. However, an interesting finding of FL blue shift due to long time preserving protein is demonstrated in this section, which finally changed the original plan, dissolving β-lactoglobulin and making solution only when it is needed. Since the purpose is to obtain the change caused by using long time preserved protein solution, VFD is not used in this step.

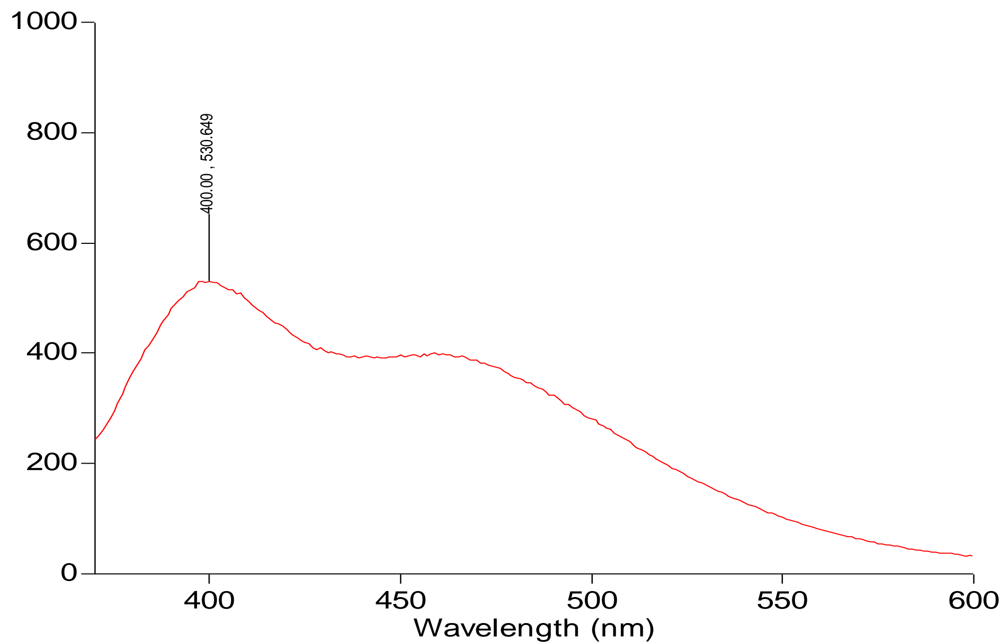


Figure 18 The fluorescence of using 4 days preserved β-lactoglobulin

Figure 18 shows the fluorescence result of 24 hours denatured protein solution labeled by TPE-MI without using VFD, the only difference with the part 4.6.2 (Figure 9A) is this experiment uses the protein stock solution after 4 days preserving. In this figure, two distinct peaks are observed, one is at the wavelength of 470nm which is expected in this experiment, the other unexpected one is at the wavelength of 400nm but the intensity is significantly higher than the intensity at 470nm.

The finding suggests that there may have some unknown substances or mechanisms happened in the protein solution after long time preserving which causes the other fluorescence peak at wavelength of 400nm. In order to further study this phenomenon, the researcher did the same experiment using the same protein stock solution after 3 more days, the result is shown on Figure 19.

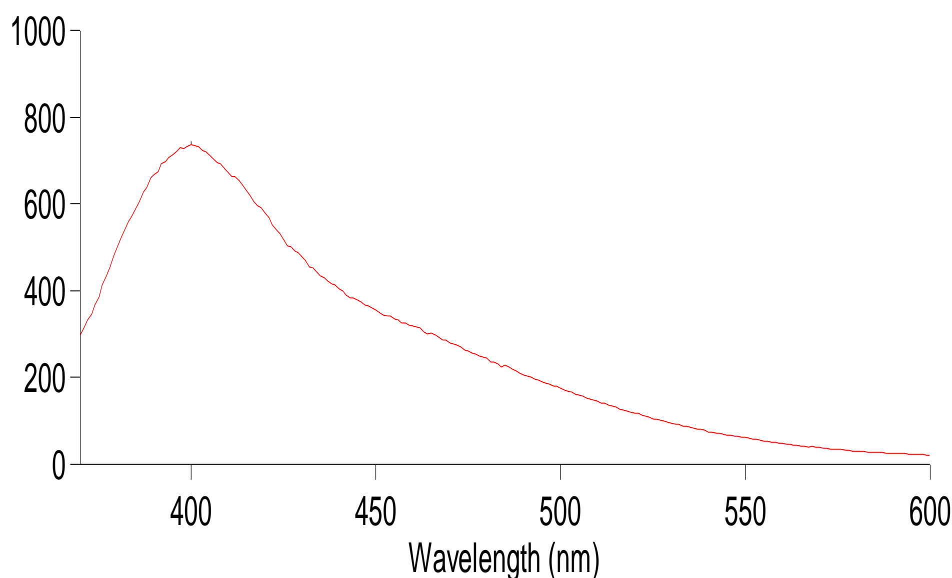


Figure 19 The fluorescence of using 7 days preserved β-lactoglobulin

Using the 7 days preserved protein stock solution, the intensity peak at 470nm disappeared, instead the intensity at 400nm increased largely to the machine unit of 700, which is remarkably higher than the final intensity obtained from 4.6.2 (Figure 9A). Since the FL intensity at 470nm is still 400 (a.u.), which is the same result as Figure 18 and similar to Figure 9A, it is supposed that the free-cysteine finished the reaction with TPE-MI and emitted fluorescence. The intensity at 400nm increased gradually from using fresh protein solution to 4 days protein solution and then 7 days protein solution indicates that there is other unknown substance or mechanism caused fluorescence due to long time preserving β-lactoglobulin, one possibility is that other bio-thiol are exposed as the protein solution were keeping in the fridge since the MI group in TPE-MI is thiol reactive, but it will certainly bring challenge to the specificity of TPE-MI, which is already identified in the previous research.

Blue shift to the wavelength of 400nm phenomenon was also discovered in a previous study (Das A et al. 2020), which the researchers used TPE-TPP to identify three aggregation intermediates of amyloid-β. The result indicates that the emission wavelength transition represents the aggregation state change of amyloid-β. Even though this research also used an AIE dye to label amyloid, the author is not confident to say the peak intensity at 400nm is the result of unfolded β-lactoglobulin aggregation and cannot relate it to the stock solution preservation time, this may leave for the future work of this protect.

Chapter 6 Discussion:

As the improving of people's life quality, the diseases caused by bad sanitation and unexpected infection can be avoided nowadays, whereas the diseases due to heredity or gene variation are still difficult to be prevented, such as Huntington's disease, the proteins tend to misfold and aggregate because of gene mutant, finally the misfolded proteins accumulation will cause the loss function of the patient's brain and eventually lead to death, according to statistic, Australia has over 1800 people suffering from Huntington's disease in 2017 and more than 9000 are at risk. This research aims to provide some new ideas from both treatment aspect and diagnosis aspect.

People have done plenty of researches on how to modify the protein structure in vitro. The procedure of these researches is usually starting from acquiring inclusion body from the body cell, then solubilizing the inclusion body, the commonly used methods include heat shock and adding high concentration of chaotropic agent such as GuHCl and urea. Finally, refolding techniques include dilution, dialysis and chromatography. However, the purpose of these methods are all for agent removal. In this thesis, VFD is a creative way because it uses high shear stress provided by the high rotational speed, without removing the denaturant agent, it can accomplish the purpose of refolding proteins. The efficiency of refolding using VFD has been identified nearly 30% of the total denatured proteins, considering the short time of VFD using, the efficiency is remarkable. Comparing to the traditional ways of refolding protein, VFD has the advantages of rapid response, low cost and easy to control. VFD was also used during the denaturation process in this project, due to the more efficient mixture caused by high rotational speed the denatruation speed is increased, even though taking refolding mechanism into the consideration the reaction promoting is promising, but the author does not suggest using VFD to accelerate the reaction because the actual increment is tiny.

It is reasonable to think that the efficiency of VFD refolding proteins can be improved because the rotational speed was fixed in this project. As mentioned in the thesis previously, the different rotational speed will result different fluid behaviour within the VFD tube, for the fluid that main component is water, 5000rpm can make it form to a double helix, which is theoretically more efficient than single helix (3000rpm) and irregular shape (4000rpm). However, for the sample used in this project, the situation will be much different with the fluid just contains water because ions and macromolecule are present in the solution, additionally, since the experiment that rotational speed higher than 5000 rpm has not been conducted, so

that there are still unknowns need to be discovered in the future work.

In general, the author believes VFD is a high potential technology that need to be further developed, using VFD to refold proteins has provided a new direction of manipulating protein structure. It is promising that this technology is being used for amyloidsis treatment in the future.

Fluorescence and circular dichroism are used for monitoring the protein structure changes caused by VFD. In summary, it is found that circular dichroism can give researchers more information regarding the protein structure, whereas the fluorescence method focuses on the intensity at the certain wavelength, it gives the information only on the content of specific substance, which is unfolded protein in this case.

The commonly used diagnostic methods for amyloid diseases are X-ray and MRI, but no biochemical methods have been reported before. Given on the advantages of simplicity of operation, affordable and easy to analyze, the author wishes to develop fluorescence for a novel method of detecting amyloidosis. The flaw of this project is that it used the pure commercial protein instead of the protein from human body. Even though the sensitivity and specificity are identified in the previous study, which proves the result can not be influenced by the other ions or thiols in cells, the sample collection is still a necessary step when using it in clinic, since the different amyloidosis will result the misfolded protein aggregation in different organs. Therefore, the author proposes that TPE-MI can be used to target the specific body cells in the future work, for example, for the detection of Huntington's disease, brain cells should be involved. By observing the fluorescence intensity due to TPE-MI labeling unfolded protein within the specific body cells, the author believes it is possible to diagnose amyloidosis using biochemical method in the future.

An interesting phenomenon was found in this project, after long time preserving of the protein β-Lactoglobulin, the fluorescence caused by denaturation and TPE-MI labeling blue shifted from the wavelength of 470nm to 400nm. In a previous study, blue shifting to 400nm was also found to happen when an AIE dye labels proteins, the researchers explain that by the aggregation of proteins during the process. However, the situation is different with in this project, when using fresh protein solution, the blue shift did not happen, only happens when the protein is long time preserved. In author's opinion, the fluorescence intensity at 400nm is indeed because the aggregation of proteins as the previous research says, when the protein solution is kept long time, it is much easier that aggregation happens. This hypothesis will have to be further proof in the future.

Chapter 7 Conclusion:

This project is based on the findings of two main previous researches, one is the fluorescence dye synthesized by an AIE core TPE and a MI group can specifically bond with the free-cysteine, which is exposed when the protein is on the unfolded state, then results in high fluorescence intensity and possess the aggregation-induced emission phenomenon; the other research finding is using a vortex fluidic device can effectively make the unfolded protein back to folding state, it has the advantage of rapid response, low cost and easy to control comparing to the traditional ways of refolding proteins. By combining the two findings, this research aims to manipulate the protein structure using VFD and monitor the process using CD and AIE fluorescence, creatively, VFD was also used during the denaturation process. Despite identifying and optimizing the new methods, the significance of this project is providing more possibility on the amyloidosis detection and treatment, which is the diseases caused by aggregation of unfolded proteins,

The project started from experiment preparation, the measuring signal establishment and setting essential experiment conditions. The data of CD measurement suggests that the protein β-Lactoglobulin folding extent has distinct influence on the ellipticity at the wavelength of 216nm. The protein possesses more complete structure has negatively higher ellipticity, which means more β-sheet content. The fluorescence signal is at wavelength of 470nm after the sample being excited at 350nm, the higher fluorescence intensity indicates that more free-cysteine has bonded with TPE-MI, in the other word, more proteins are unfolded. The experiment condition setting including denaturant concentration and AIE labeling time, although other settings can also vary the experiment result such as protein concentration and VFD rotational speed, since they are already reported in the previous studies, the author decides to follow these settings in this project. Determining the denaturant concentration requires two conditions, one is the denaturation process finishes after 24 hours because of loss efficient of denaturant and the other one is the reaction is still going on after 3 hours, finally the denaturant concentration of 4M is used. AIE dye needs time to label with unfolded protein in order to detect the fluorescence, after making sure that there is no more unfolded protein produced after 24 hours, the experiment found that after 2.5 hours, the fluorescence intensity tends to be stable.

Refolding protein using vortex fluidic device has been reported by the previous studies, in this project, this phenomenon has been proven again by measuring with CD and AIE fluorescence. Moreover, this project has tried using VFD during denaturation process, the original thought

was to provide a new idea of stopping protein denaturation. However, the result indicates that when the denaturant is still present in the protein solution, using VFD can not reverse the denaturation process, even slightly promote it. If taking the VFD refolding protein mechanism into consideration, the promoting result is very significant. Yet the author does not suggest using VFD to increase the denaturation speed while there is still enough denaturant present, because the actual increment is tiny.

Two measuring methods were used and compared in this project, CD and AIE fluorescence. The difference of the two methods can be sorted by the source of the incident light, CD uses circularly polarized light and fluorescence uses non-polarized light; or from the mechanism aspect, CD is due to the different absorption of a molecule to left-hand circularly polarized light and right-hand circularly polarized light, fluorescence is detected because the molecule can emit light at a certain wavelength after being excited. With no doubt, in general, the CD measurement gives more information than fluorescence measurement because it measures a range of wavelength, the different optical chiral molecules within a protein result ellipticity at different wavelength, however, for fluorescence, since the emission wavelength of a excited molecule is specific, the resulting intensity at the certain wavelength is the only thing that needs to be concerned. Speaking for this project, the author believes that using AIE fluorescence method to detect and monitor the protein folding state is easier, cheaper and more direct comparing to CD. One thing has drawn the author's attention is that at present, the diagnostic approaches of the diseases related to unfolded proteins are mainly X-ray and MRI, biochemical methods are rarely used, hopefully this AIE fluorescence can provide a new possibility of diagnosis in the future.

References

1. Berde CB, Hudson BS, Simoni RD & Sklar LA, 1979. "Human Serum Albumin". The Journal of Biological Chemistry. Vol.254, No.2, pp.391-400.
2. Britton J, Smish JN, Raston CL & Weiss GA, 2017. "Protein Folding Using a Vortex Fluidic Device". Methods Mol Biol. No. 1586, pp.211-220.
3. Britton J, Stubbs KA, Weiss GA & Raston CL, 2017. "Vortex Fluidic Chemical Transformation". ChemPubSoc Europe. DOI: 10.1002, pp.13270-13278.
4. Chen MZ et al., 2017. "A thiol probe for measuring unfolded protein load and proteostasis in cells". Nature Communications. No. 8: 474, DOI: 10.1038.
5. Chen et al., 2017. "Quantitation urinalysis using aggregation-induced emission bioprobes for monitoring chronic kidney disease". Faraday Discussions. No.196, pp.351-362.

6. Chemistry LibreTexts, 2019. Circular Dichroism.
7. Das A, Gupta A, Hong Y et al., 2020. "A Spectroscopic Marker for Structural Transitions Associated with Amyloid-β Aggregation". Biochemistry, Vol. 59, pp. 1813-1822.
8. Ding S, Liu M & Hong Y, 2018. "Biothiol-specific fluorescent probes with aggregation-induced emission characteristics". SCIENCE CHINA Chemistry. Vol.61, No.8, pp.882-891.
9. Dobson CM, 2003. "Protein folding and misfolding". Nature. Vol.426, pp.884-890.
10. Eftink MR, 1991. "Fluorescence Techniques for Studying Protein Structure". METHOD OF BIOCHEMICAL ANALYSIS. Vol.35
11. GE Healthcare, "Protein Sample Preparation"
12. Gao M & Tang B, 2017. "Fluorescent Sensors Based on Aggregation-Induced Emission: Recent Advances and Perspectives". ACS Sensors. DOI:10.1021
13. Hong Y, Lam J & Tang B, 2011. "Aggregation-induced emission". Chem. Soc. Rev. Vol.40, pp.5361-5388.
14. Hong Y et al., 2010. "Quantitation, Visualization, and Monitoring of Conformational Transitions of Human Serum Albumin by a Tetraphenylethene Derivative with Aggregation-Induced Emission Characteristics". Anal.Chem. Vol.82, pp.7035-7043
15. Huntington's NSW ACT, 2020. "How common is Huntington's Disease (HD)?"
16. Kelly SM & Price NC, 1997. "The application of circular dichroism to studies of protein folding and unfolding". Biochemica et Biophysica Acta. No.1338, pp.161-185
17. Lgder A, Pye S et al., 2020. "Vortex fluidic mediated synthesis of polysulfone". Royal Society of Chemistry. October, pp. 14761-14767.
18. Lobley A. Whitmore L. & Wallace B.A. 2002. "DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra". Bioinformatics 18, pp. 211-212.
19. Luo J, Xie Z, Lam J. W. Y et al., 2001. "Aggregation-induced emission of 1-methyl-1,2,3,4,5-pentaphenylsilole". CHEMCOMM Communication. 19th June.
20. Luo X, Jarvey D, Ruan Y et al., 2018. "Vortex Fluidic Mediated Synthesis of Macroporous Bovine Serum Albumin-Based Microspheres". ACS APPLIED MATERIALS & INTERFACES. pp.27224-27232.
21. Liu Y et al., 2010. "Simple Biosensor with High Selectivity and Sensitivity: Thiol-Specific Biomolecular Probing and Intracellular Imaging by AIE Fluorogen on a TLC Plate through a Thiol-Ene Click Mechanism". Chem. Eur. J. Vol.16, pp.8433-8438.
22. Mao D., Wachter E. & Wallace B.A., 1982. "Folding of the Mitochondrial Proton Adenosinetriphosphatase Proteolipid Channel in Phospholipid Vesicles". Biochemistry, 21, pp. 4960-4968.
23. Matsuura E. J. & Manning C. M, 1994. "Heat-Induced Gel Formation of β-lactoglobulin: A Study on the Secondary and Tertiary Structure As Followed by Circular Dichroism Spectroscopy". J. Agric. Food Chem. Vol. 42, pp.1650-1656.

24. Ross CA et al., 2014. "Huntington disease: natural history, biomarkers and prospects for therapeutics". Nature. Vol.10, pp.204-216.
25. Shen P et al., 2017. "Recognition and quantification of HSA: A fluorescence probe across alpha-helices of site I and site II". Sensors and Actuators. No.247, pp.587-594.
26. Sreerema, N., Venyaminov, S.Y. & Woody, R.W., 1999. "Estimation of the number of helical and strand segments in proteins using CD spectroscopy". Protein Sci. 8, pp. 370-380.
27. Sreerema, N. & Woody, R.W., 2000. "Estimation of protein secondary structure from CD spectra: Comparison of CONTIN, SELCON and CDSSTR methods with an expanded reference set". Anal. Biochem. 287(2), pp. 252-260.
28. Tong H et al., 2007. "Protein Detection and Quantitation by Tetraphenylethene-Based Fluorescent Probes with Aggregation-Induced Emission Characteristics". J. Phys. Chem. B. No.111, pp.11817-11823
29. ThermoFisher. "Fluorescence Fundamentals"
30. Vimalanathan K & Raston CL, 2017. "Dynamic Thin Films in Controlling the Fabrication of Nanocarbon and Its Composites". Advanced Materials Technologies. No.1600298.
31. Yuan TZ et al., 2015. "Shear stress-mediated refolding of proteins from aggregates and inclusion bodies". Chembiochem. Vol.16(3), pp.393-396.
32. Vonsattel JPG & Difiglia M, 1998. "Huntington Disease". Journal of Neuropathology and Experimental Neurology. Vol.57, No.5, pp.369-384.
33. Whitmore L. & Wallace B.A. 2008. "Protein Secondary Structure Analysis from Circular Dichroism Spectroscopy: Methods and Reference Databases". Biopolymers 89. pp. 392-400.