Doctoral Dissertation

Design the polyampholytes in inhibiting protein aggregation and exploring its mechanism

XIANDA DAI

Supervisor: Kazuaki MATSUMURA
Graduate School of Advanced Science and Technology
Japan Advanced Institute of Science and Technology
Materials Science

March 2025

Abstract

Protein plays a vital role in human activities. However, environmental factors often lead to protein aggregation, which has a serious impact on human health. In addition, certain protein drugs, such as insulin, also lose their efficacy due to degenerative aggregation and can even be life-threatening. Therefore, the inhibition of protein aggregation has become an important problem to be solved.

In this study, developed a polyampholytes electrolyte composed of ε-polylysine and succinic anhydride, and comprehensively evaluated its protein protective effect. The experimental results show that this polymer can effectively protect a variety of proteins from thermal stress damage, and its effect is significantly better than that of previously reported zwitterionic polymers. In addition, I have synthesized derivatives with different hydrophobicity, further improving their protection efficiency. In particular, the polymer concentration required to achieve protein protection is extremely low. By promoting the retention of protein enzyme activity and stabilizing higher-order structures, these polymers allow proteins to remain in their natural state even after being subjected to extreme thermal stress.

Therefore, this polyampholytes electrolyte performs well in protecting proteins from extreme stress and has a wide range of applications, especially in protein biopharmaceutical and drug delivery systems. In addition, the synthesis process of the polymer is simple, low cost, and can play a good protective role at very low concentration, which lays a solid foundation for its large-scale application.

[Keyword] Polyampholytes; hydrophobicity; protein protection; surface charge; low concentration.

Contents

Chapter 1

Introduction

1.1 Protein and protein aggregation

The chemical structure of proteins is similar to that of polymers, which are composed of polypeptide chains with specific amino acid sequences. These polypeptide chains are arranged and folded in precise ways to form more complex protein structures. The basic components of proteins include carbon, hydrogen, oxygen and nitrogen, which ensure the structural stability and functional diversity of proteins through the interaction of chemical bonds such as covalent bonds and hydrogen bonds. In some proteins, the addition of other elements such as sulfur and phosphorus further enrich the function and properties of proteins.

As the material basis of life, protein is not only a kind of organic macromolecule, but also the basic organic matter constituting the cell. They play a variety of key roles in the cell, such as enzyme catalysis, antibody immune defense, hormone signaling and so on. The diversity and complexity of proteins make them the main performers of life, from metabolic processes within cells to signal communication between cells. It can be said that without protein, life would be impossible. The processes of protein synthesis, folding, modification and degradation are all necessary for life to maintain normal physiological functions. The misfolding or abnormal function of proteins often leads to the occurrence of various diseases, such as Alzheimer's disease and Parkinson's disease. Therefore, the in-depth study of protein structure and function is of great significance for understanding life phenomena, developing new drugs and treating diseases.

With the vigorous development of science and technology, the application fields of protein are increasingly extensive, and its importance is becoming more and more prominent. In many cutting-edge fields such as bioengineering, modern biochemistry, and pharmaceuticals, proteins not

only play a key role, but also become the core force driving technological innovation.[1] The continuous expansion of these fields not only highlights the diversity and functionality of proteins, but also indicates that proteins will play a more important role in the development of science and technology in the future.

In recent years, as a new therapeutic method, protein drugs have gradually emerged in various fields and attracted much attention. Its significant advantages are mainly reflected in the following aspects: First, the basic materials of protein drugs are easy to obtain, providing a solid foundation for large-scale production. Secondly, such drugs are not only effective, but also have very low side effects, avoiding the accumulation of poisoning problems that may be caused by traditional drugs. In addition, the application range of protein drugs is extremely wide, there are many varieties, and the development of new drugs is endless, which provides a rich choice for clinical treatment. Finally, the research and development process of protein drugs has a clear goal and specificity and can accurately solve specific disease problems. In summary, with its unique advantages, protein drugs are gradually becoming an important pillar in the field of modern medicine.

In addition, it is worth noting that certain protein drugs show significant therapeutic potential when used in combination with antibody therapies. Remarkable results have been achieved in the treatment and control of many diseases such as cancer, allergy and autoimmune diseases.[2]

One well-known protein drug is insulin (Figure 1.1). Since the 1980s, the global prevalence of diabetes has nearly doubled from 4.7% to 8.5% in just over 30 years. At the same time, the number of people with diabetes also rose sharply, from 108 million to 422 million.[3] Insulin, a key protein hormone, is precisely regulated by the beta cells of the human pancreas. In response to specific substances such as glucose, lactose, and glucagon, beta cells secrete insulin. Insulin in the body with its unique ability to lower blood sugar levels, while promoting fat, protein and glycogen synthesis, maintaining the body's metabolic balance plays a vital role.[4] The accumulation of insulin degeneration leads to the loss of biological activity. This phenomenon is

particularly common in people with diabetes, especially at the site of frequent insulin injections, where the incidence of insulin amyloidosis is significantly increased.[5] The aggregation of insulin not only affects the therapeutic effect, but also leads to the aggregation of proteins. This aggregation may not only trigger the body's immune response, but even in severe cases, may endanger our lives. Another important protein drug, Herceptin® (Figure 1.1), has been widely used to treat metastatic breast cancer. As a targeted therapy, Herceptin has significantly improved the survival rate of cancer patients and brought hope to countless patients. However, the stability of Herceptin cannot be ignored. Studies have shown that Herceptin is prone to aggregation under conditions such as high pH and temperature. Once the antibodies accumulate, their efficacy will be greatly reduced, or even completely ineffective, so that they can no longer provide effective treatment for patients.[6]

(a) **(b)**

Figure 1.1. (a) The picture of the insulin.[7]. (b) The picture of the Herceptin®[8]

As mentioned earlier, proteins are prone to irreversible aggregation and degeneration, which not only significantly impair their biological activity, but can also lead to a range of serious diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease.[9,10] Protein aggregation has a significant effect on the potency of protein drugs. Therefore, how to effectively inhibit protein aggregation has become a key problem to be solved.

Protein aggregation refers to the process by which misfolded proteins gradually converge into aggregates and organized fibril through specific conformations (Figure 1.2). This phenomenon can be induced by a variety of factors, such as high temperature, high pH, and mechanical shaking.

Figure 1.2 The picture of the protein aggregation.[11]

1.2 Mechanism and factors of protein aggregation

To effectively address the side effects of protein aggregation, we first need to deeply understand its formation mechanism and induction factors. Many studies have revealed that the main driving factors of protein aggregation include its own mutation, external environment stimulation, and the imbalance of protein homeostasis in the cell. These factors play a crucial role in the process of protein aggregation, so it is of great significance to fully analyze the mechanism of action of these factors for formulating effective intervention strategies.[12]

Mutations in the protein itself are often caused by changes in the gene sequence, leading to abnormalities in the structure and function of the protein. This mutation may make the protein more prone to misfolding, which in turn promotes the formation of aggregates.[13] External environmental stimuli, such as oxidative stress, temperature changes, pH abnormalities, etc., can also significantly affect the stability and folding state of proteins, thereby inducing aggregation. In addition, an unbalanced response to intracellular protein homeostasis, such as dysfunction of the protein degradation system or an abnormal increase in the rate of protein synthesis, can lead to the accumulation of unfolded or misfolded proteins, eventually triggering aggregation.[14]

Further research shows that these driving factors do not exist in isolation but interact and influence each other. For example, protein mutations may make cells more susceptible to external environmental stimuli, which in turn may exacerbate imbalances in protein homeostasis within cells.[15] Understanding the interrelationships between these factors is therefore essential to fully uncover the complex mechanisms of protein aggregation.

Because proteins are made up of more than 20 different amino acids in a specific order, the human body may contain thousands of different types of proteins. Each protein has a specific function, controlling various chemical reactions in our body to maintain normal life activities. In vivo, protein synthesis is a complex and delicate process. Normally, newborn proteins need to go through a series of precise folding steps to form their

characteristic three-dimensional structure, so that they can properly perform their biological functions. However, when proteins encounter adverse factors such as aging, mutation, or environmental upheaval, the folding process tends to go awry. This misfolding not only results in a loss of protein function but can also trigger protein aggregation or the formation of amyloid, which can cause serious damage to cells and even entire organisms. Therefore, maintaining the correct folding state of proteins is essential for maintaining the normal operation of life activities. [16,17,18,19]

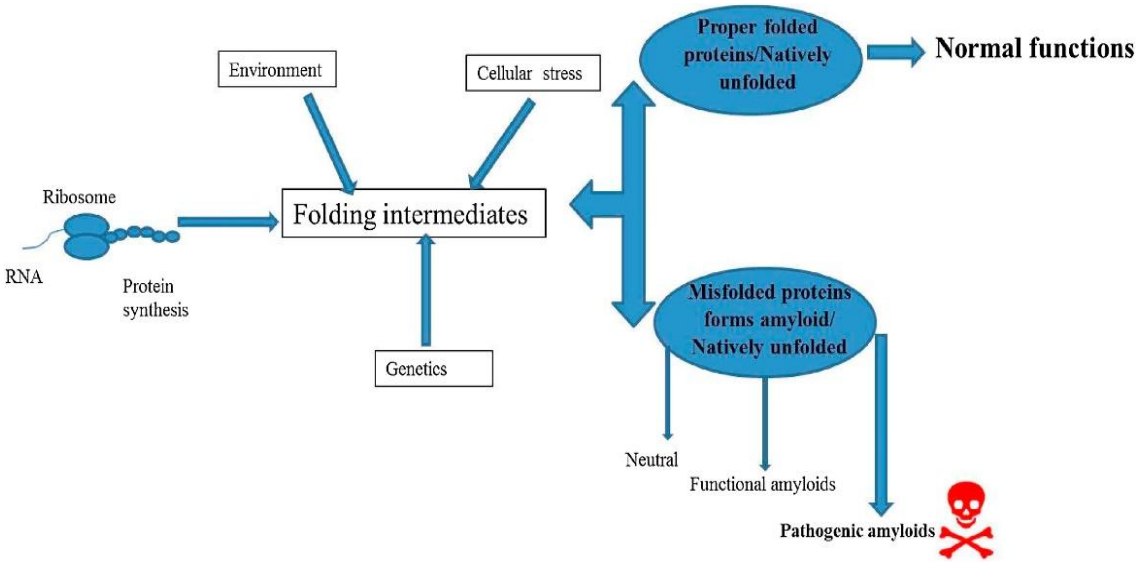


Figure 1.3 The picture of the protein aggregation.[20]

In vivo, protein aggregation can be induced by a variety of factors, including pathogenic mutations, protein overexpression, defects in synthesis, protein autophagy induced by environmental stress, proteasome damage, and natural protein aging.[18] In addition, mutations in human proteins not only lead to misfolding and aggregation of related proteins, but also can lead to a range of protein misfolding diseases, such as Huntington's disease, type 2 diabetes, familial Parkinson's disease, and Alzheimer's disease.[21,22] The occurrence of these diseases reveals the key role of protein aggregation in the pathological process, and further highlights the importance of in-depth research on the mechanism of protein homeostasis maintenance.

In vitro, a variety of environmental factors can induce protein aggregation. These factors include, but are not limited to, pH, temperature and salt concentration, which contribute to the aggregation of proteins by affecting their structural stability.

The effect of pH on proteins is mainly reflected in two aspects. Studies have shown that proteins exhibit dipole-like properties near their isoelectric points due to the balanced distribution of positive and negative charges. This property not only enhances the mutual attraction between protein molecules, but also provides favorable conditions for the formation of aggregates.[23] Inside the protein, changes in pH can trigger a series of adverse effects. The specific function of proteins depends on their unique spatial folding structure, which is maintained by intermolecular forces within the protein molecule. Therefore, when the pH value in the environment changes significantly, the electrostatic force inside the protein molecule is affected, which in turn leads to changes in the molecular structure, and ultimately the loss of its unique function.

Temperature is also a key factor that cannot be ignored. The right temperature helps maintain the natural conformation of the protein, while too high or too low a temperature may lead to protein denaturation or conformational instability, which promotes aggregation. At high temperature, protein molecular movement intensifies, hydrophobic groups are exposed, and intermolecular collision frequency increases, all of which are conducive to the formation of aggregates. In contrast, low temperatures can slow down the denaturation process of proteins, but in some cases may also indirectly promote aggregation by reducing the solubility of proteins.

In addition, inside the protein, due to the existence of intermolecular interactions between amino acid residues of different molecules, attraction is sometimes triggered, which leads to the aggregation of proteins. These interactions include hydrophobic interaction, hydrogen bond interaction and electrostatic interaction. These interactions not only interfere with the normal arrangement of amino acid residues, but also encourage undesired binding to occur, ultimately leading to protein misfolding.

Hydrophobic interactions are caused by the tendency of non-polar amino acid residues to cluster together in a water environment to reduce contact with water molecules, thereby reducing the free energy of the system.[24] This aggregation trend is particularly pronounced in protein molecules, especially in the interior of proteins, where the aggregation of non-polar residues contributes to the formation of a stable protein core. However, when this hydrophobic interaction occurs at the wrong place on the protein surface, it can lead to misfolding of protein molecules and even trigger aggregation.

Hydrogen bond interaction is another important stabilizing factor in protein structure. Hydrogen bonds are caused by the uneven distribution of charge between hydrogen atoms and electronegative atoms such as oxygen or nitrogen. In proteins, hydrogen bonds exist mainly between polar amino acid residues, such as asparagine, glutamine, serine, and threonine. These hydrogen bonds play a key role in maintaining the secondary and tertiary structures of proteins.[25] However, when the location or direction of hydrogen bond formation is wrong, the normal folding process of the protein can be disrupted, resulting in the loss of functional proteins.

Electrostatic interactions are caused by charge interactions between charged amino acid residues such as lysine, arginine, aspartate, and glutamate.[26] Under physiological conditions, electrostatic interactions between these charged residues can significantly affect the structure and stability of proteins. For example, attraction between positively and negatively charged residues can stabilize the structure of a protein, but when these interactions occur in the wrong place, they can lead to misfolding and aggregation of proteins.

In summary, the aggregation of proteins is mainly due to the improper occurrence of hydrophobic interaction, hydrogen bond interaction and electrostatic interaction. These interactions not only interfere with the normal arrangement of amino acid residues, but also encourage undesired binding to occur, ultimately leading to protein misfolding. Understanding the mechanisms of these interactions has important implications for the prevention and treatment of diseases associated with protein misfolding,

such as Alzheimer's and Parkinson's diseases. By regulating these intermolecular interactions, I expected to develop new strategies to maintain the normal function of proteins, thereby promoting human health.

1.3 The ways of Inhibit the protein aggregation

1.3.1 Polyphenol

So far, scientists have found a lot of ways to suppress the aggregation of proteins and have made great progress. Polyphenols, a compound that is widely found in plants, have received much attention due to their unique chemical structure and potential health benefits.[27] The core feature of polyphenols is that their molecules contain multiple phenolic groups, which give them significant antioxidant properties.[28] Antioxidant effects are one of the most touted health benefits of polyphenols, which can neutralize free radicals in the body and reduce oxidative stress, thus helping to prevent a variety of chronic diseases, such as cardiovascular disease, cancer and neurodegenerative diseases.

More importantly, research in recent years has further revealed the remarkable efficacy of polyphenols in inhibiting protein aggregation. [29] Protein aggregation, especially abnormal aggregation, is a common pathological feature of many neurodegenerative and aging-related diseases. These aggregated proteins not only lose their normal biological function but may also form toxic substances that further aggravate the disease process.

Turmeric, a perennial plant of the ginger family, whose roots are dried and ground to transform it into a valuable spice, is used in many Indian dishes and curries. Not only that, turmeric also with its natural yellow pigment properties, has been cleverly used in the field of food processing, becoming a unique edible yellow dye.[30] In South Asia, turmeric has been widely used since ancient times to treat a variety of human diseases, and it is not only a central ingredient in many Ayurvedic and Unani pharmaceutical preparations, but also occupies an integral position in traditional medicine. [30]

Curcumin, a low molecular weight polyphenolic compound, is derived from turmeric and is known for its unique yellow pigment. (Figure 1.4)

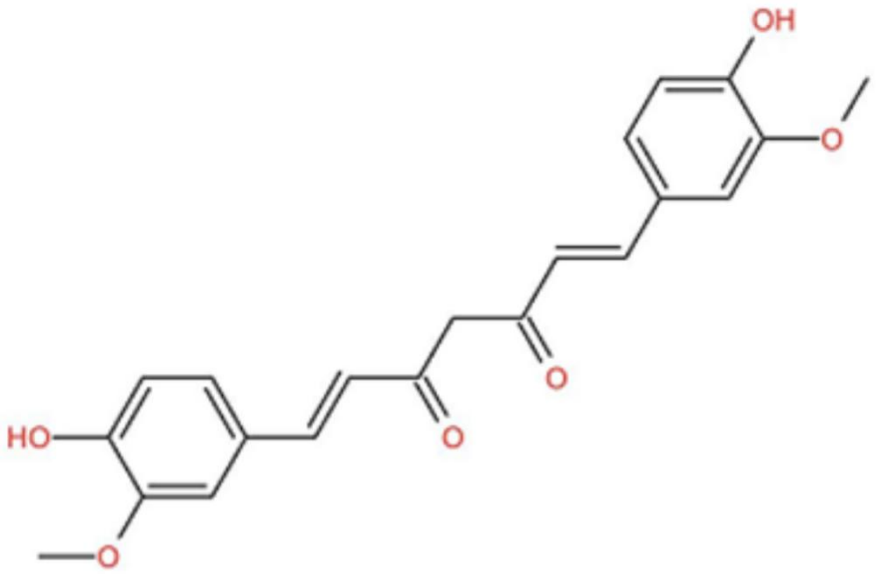


Figure 1.4 The chemical structure of the curcumin.

Curcumin, due to its unique chemical structure, exhibits a high degree of plasticity, which allows its molecules to flexibly adapt to appropriate conformations based on hydrophobic interactions. This property plays a key role in inhibiting protein aggregation. Specifically, curcumin binds tightly to the protein through these hydrophobic interactions, thereby stabilizing the three-dimensional folded structure of the protein and effectively preventing the denaturation of the protein.[31]

The mechanism of action of curcumin is based on this principle. Hydrophobic groups in its molecular structure can interact with hydrophobic regions on the protein surface to form stable complexes. This binding not only prevents the misfolding of the protein, but also promotes the correct folding of the protein, thus maintaining the natural conformation of the protein.

In recent years, scientists have conducted in-depth research on the potential role of Curcumin in inhibiting Aβ aggregation. Ono et al. were the first to demonstrate that curcumin exhibits a dose-dependent inhibition of A β42 and Aβ40 fibril formation.[32]

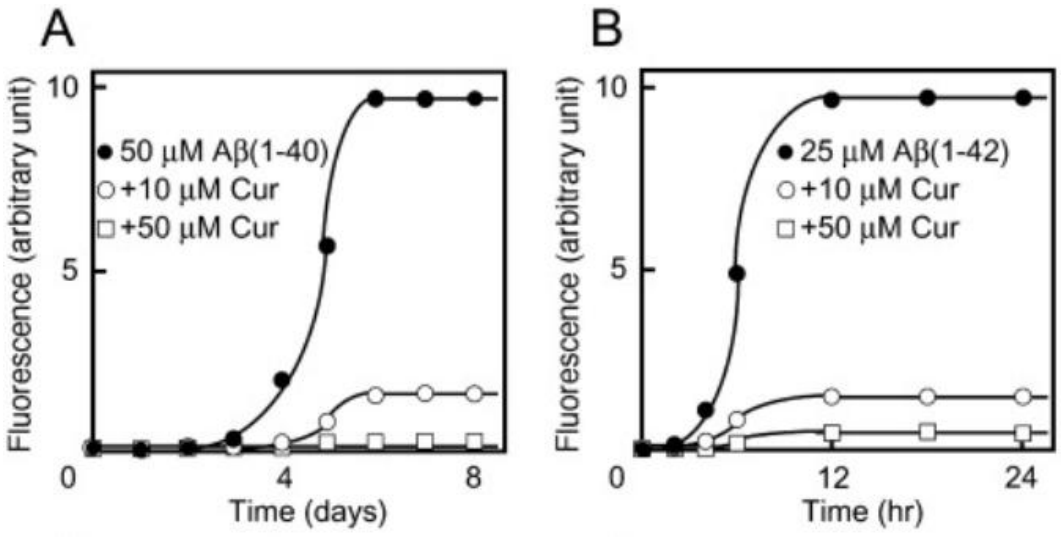


Figure 1.5 Effects of Cur (A,B) on the kinetics of formation of fAβ(1–40) (A) and fA β(1–42) (B) from fresh Aβ(1–40) and Aβ(1–42), respectively.[32]

It can be seen from the figure 1.5 that the fluorescence intensity of proteins Aβ40 and Aβ42 without curcumin was significantly increased after heating, indicating that protein aggregation began. However, the fluorescence intensity of the protein supplemented with curcumin was significantly reduced after heating, indicating its unique potential in inhibiting protein aggregation.

In another study, Steven S.-S. Wang showed the great potential of curcumin in inhibiting lysozyme aggregation. [33] (Figure 1.6)

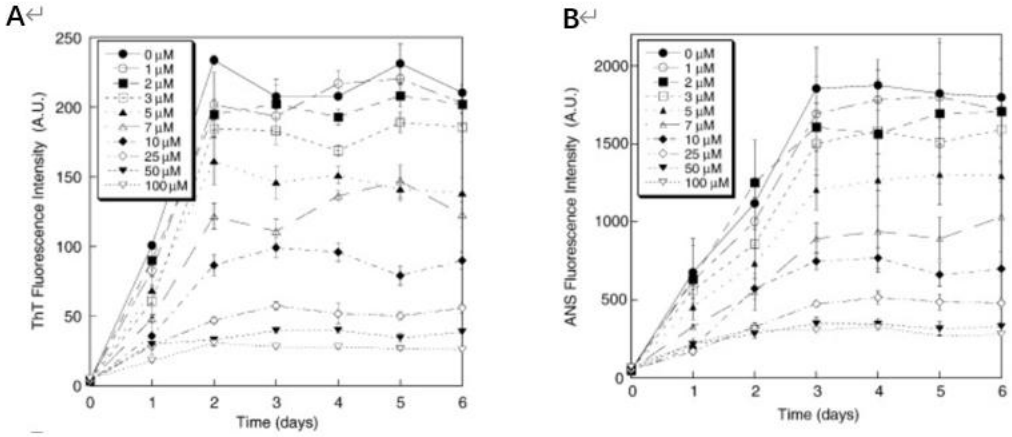


Figure 1.6 ThT (A) and ANS (B) detection of curcumin inhibiting lysozyme aggregation.[33]

As can be seen from the figure, both ThT and ANS of lysozyme without curcumin showed high fluorescence intensity after incubation for a few days, indicating that the structure of the protein had changed and was bound to the fluorescent reagent. However, the fluorescence intensity of lysozyme added with curcumin decreased significantly after heating. It showed that curcumin had a unique advantage in inhibiting protein aggregation.

Over the years, many experiments have fully proved the excellent performance of curcumin as a protein inhibitor, and its identity as a natural pigment gives it safe and non-toxic characteristics and will not cause any additional burden on the human body. However, curcumin has also exposed some significant limitations in its practical application, such as low solubility, poor stability, fast metabolic rate and short half-life. These deficiencies directly affect its bioavailability, thus limiting its wide application in the medical field to a certain extent.

For example, Fei-Ping Chen, Bian-Sheng Li et al. tried to improve the stability of curcumin by using nano-complexation between curcumin and soy protein isolate (SPI) and made some progress.[34] Figure 1.7

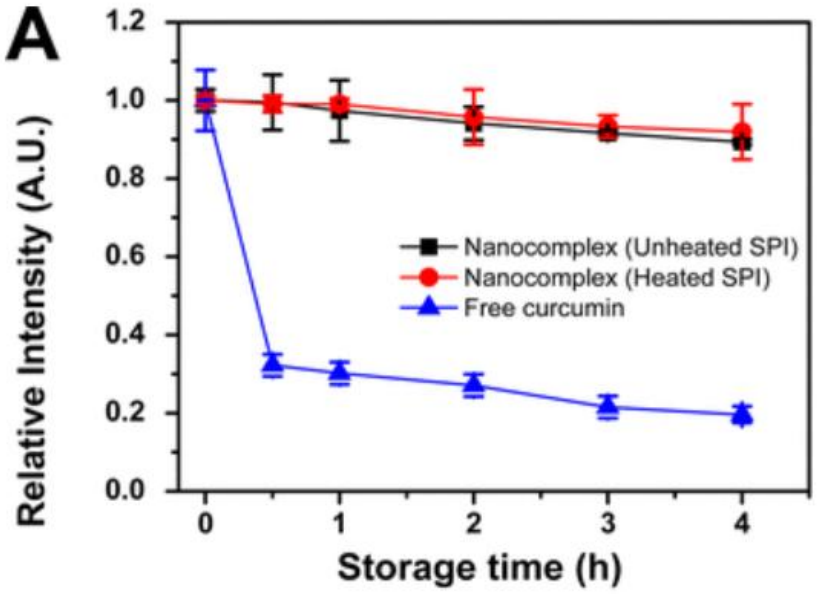


Figure 1.7 Degradation kinetics of free curcumin and curcumin in the nanocomplexes with unheated and heated (95 °C) SPIs in water at pH 7.0 upon storage for up to 4 h at 25 °C (A). [34]

1.3.2 Molecular chaperones

Inside our bodies, there are special proteins called Chaperone proteins, which play a crucial role in maintaining Protein homeostasis and function. The main function of chaperone proteins is to protect other proteins from degradation and assist them in folding properly, thus obtaining functional conformation.[35] This process is essential for maintaining the proper functioning of cells, as the correct folding of proteins directly affects the performance of their biological functions.

Through a complex set of molecular mechanisms, chaperone proteins recognize and bind to proteins that are not yet folded correctly or are in the wrong conformation.[36] They help these proteins gradually reach their correct three-dimensional structure by providing a controlled environment. Notably, although chaperone proteins play a key auxiliary role in this process, they do not themselves become part of the functional structure of the target protein.[37] In other words, the chaperone protein is temporary, and once the target protein has completed the correct folding, the chaperone protein detaches from it and continues its task of protecting and assisting other proteins.

This pattern of non-related cooperation is widespread within the cell, ensuring the correct folding of proteins and the acquisition of functional conformations. The presence and role of chaperone proteins not only prevent the misfolding and aggregation of proteins, but also reduce the risk of diseases caused by protein misfolding, such as Alzheimer's disease and Parkinson's disease.[38] Therefore, chaperone proteins have irreplaceable importance in cell life activities.

In addition, molecular chaperones not only function under normal physiological conditions, but also show higher activity under stressful conditions, such as high temperature, oxidative stress, etc.[39] This indicates that the molecular chaperone system is highly adaptable and responsive and can guarantee the correct folding of proteins in different environments, thereby protecting cells from damage.

According to relevant studies, some chaperone proteins do have the ability to help misfolded proteins refold correctly, a process that is essential for

maintaining the normal function of proteins in cells. In addition, other chaperone proteins can exert their dissolved role under certain conditions, such as when protein aggregates are formed, thereby preventing the accumulation of these harmful aggregates.[40] The expression of these functions often depends on specific stress environments, such as heat shock, oxidative stress, etc., which can induce the expression and activity of chaperone proteins.[41]

This dual role of chaperone proteins, which play a prominent role under stress while maintaining basic functions under normal physiological conditions, highlights their importance in all organisms. Whether responding to sudden environmental changes or maintaining daily cell operations, chaperone proteins ensure the correct folding and functional execution of proteins in their unique way, thus providing a solid guarantee for the survival and reproduction of organisms.

For example, Philip E. MorganTeresa M. Treweek reported on a molecular chaperone, casein.[42] Studies have shown that this molecular chaperone has great potential to inhibit insulin and Reduced α-Lactalbumin aggregation. In the experiment, dithiothreitol (DTT) was used to induce protein aggregation.

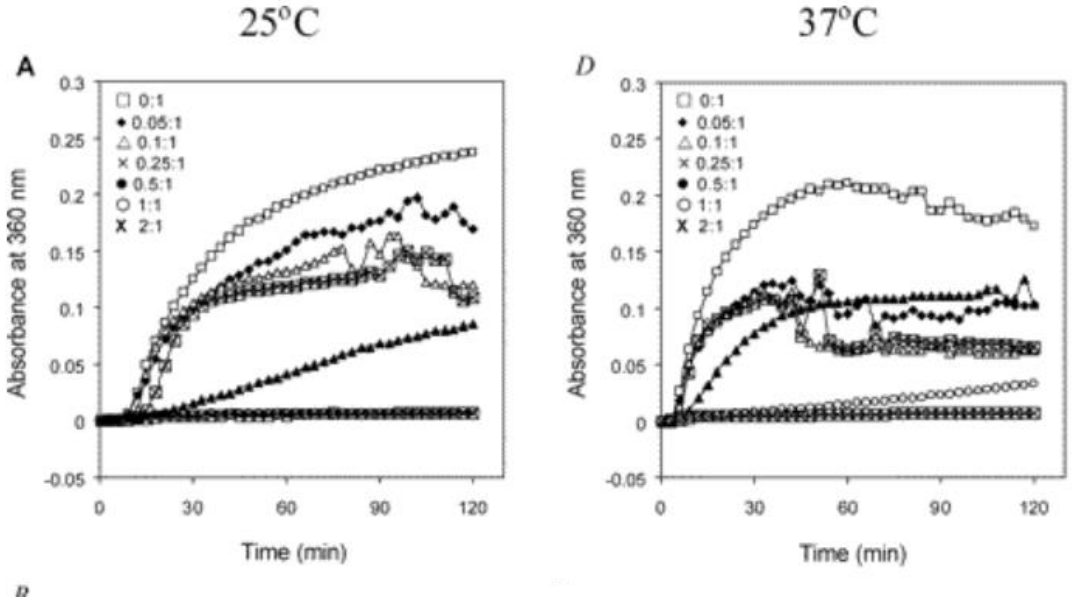


Figure 1.8 Absorbance (aggregation induced by DTT) of insulin with (different concentrations) and without casein at different temperatures. [42]

As can be seen from the figure 1.8, the absorbance of insulin without casein was significantly increased under DTT induction, indicating the aggregation of proteins. With the addition of chaperone, the absorbance of the protein solution decreased with the increase of its concentration, indicating that the presence of casein could inhibit the aggregation of insulin.

In addition, the secondary structure of another protein, Reduced α-Lactalbumin, after DTT-induced aggregation was detected by ^{1}H NMR spectroscopy. [42]

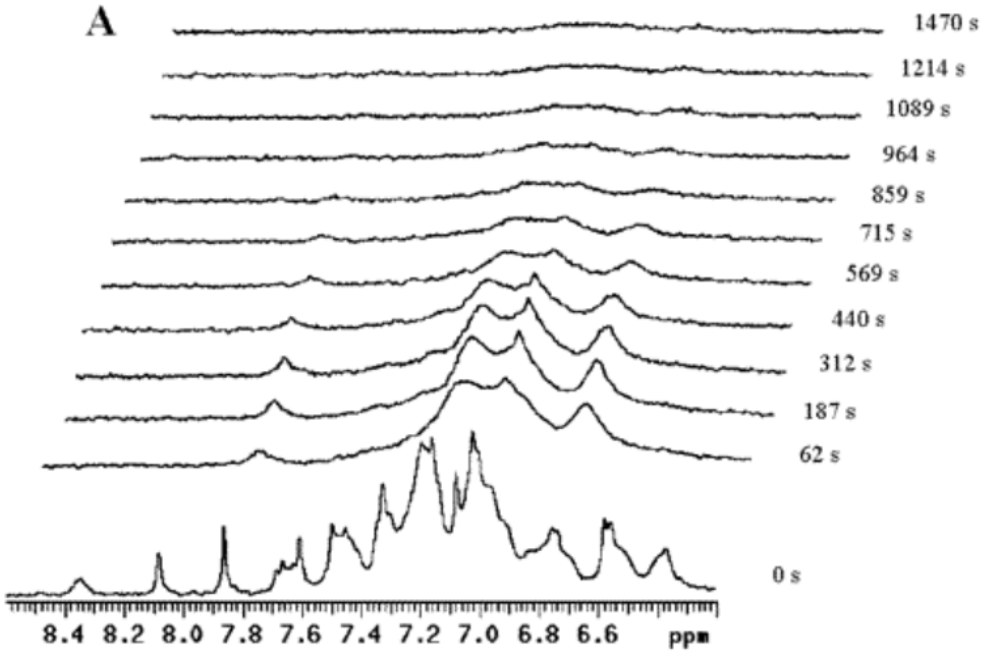


Figure 1.9 Real-time ^{1}H NMR spectroscopy of α-lactalbumin reduction by DTT at 37°C in the absence of casein. [42]

It can be seen from the figure 1.9 that after the addition of DTT, the chemical shift peaks of some amino acid residues of the secondary structure of the protein gradually disappeared with the passage of time, indicating that the secondary structure of the protein was destroyed, and the protein underwent denaturation.

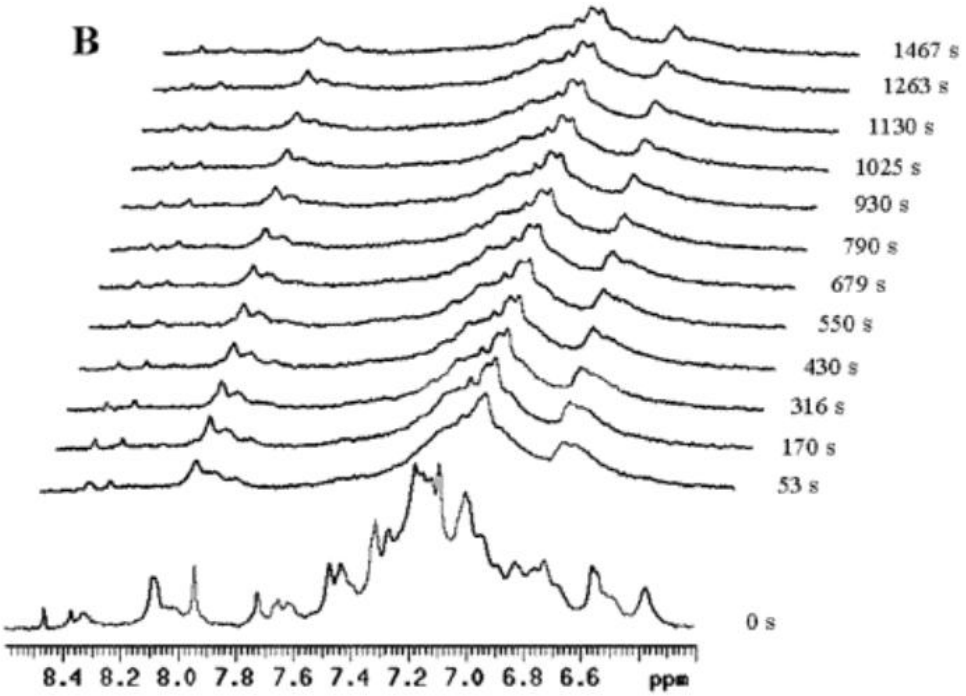


Figure 1.10 Real-time ^{1}H NMR spectroscopy of α-lactalbumin reduction by DTT at 37°C in the presence of casein. [42]

As can be seen from the figure 1.10, the chemical shift of proteins did not change significantly with the addition of molecular chaperone, indicating that the secondary structure of α-lactalbumin hardly changed significantly in the presence of casein, indicating that the secondary structure of proteins was almost intact in the presence of casein. Thus inhibiting the aggregation of proteins.

Although chaperones have shown remarkable results in inhibiting protein aggregation, there are still many challenges to their widespread application. The first problem is the specificity of chaperones, that is, a chaperone usually only works against a specific protein or group of proteins. Given the wide variety of proteins, this specificity undoubtedly limits the potential for molecular chaperones to be used in a wider variety of proteins.

In addition, the process of synthetic molecular chaperones is often complex, involving multi-step chemical synthesis and rigorous purification processes. This complexity not only increases research and development

costs but can also lead to stability issues and potential side effects in practical applications of molecular chaperones. Therefore, although molecular chaperones have significant advantages in specific fields, their scope of application is still significantly limited.

1.3.3 The zwitterionic polymers

In recent years, zwitterionic polymers have become the focus of scientific research because of their unique structures and properties. As an overall electrically neutral polymer compound, zwitterionic polymers contain both positive and negative ionic groups in the same monomer side chain within their molecules. [43] This unique molecular design gives it a series of excellent physical and chemical properties, the most notable of which is its strong hydrophilicity.

The hydrophilicity of zwitterionic polymers is mainly due to the synergistic interaction of positive and negative ionic groups in their molecular structure.[43] This synergy not only enhances the interaction between the polymer and the water molecules, but also makes it exhibit excellent solubility and stability in solutions. In addition, zwitterionic polymers also have good biocompatibility and anti-protein adsorption ability, which makes them show a wide range of application potential in the biomedical field.

In the biomedical field, zwitterionic polymers have been widely used in drug delivery systems, biomaterials surface modification and tissue engineering. For example, by combining drug molecules with zwitterionic polymers, targeted delivery and controlled release of drugs can be achieved, thereby improving therapeutic effectiveness and reducing side effects.[44] In addition, zwitterionic polymers can also be used to prepare biomaterials with antimicrobial properties, effectively reducing the risk of infection by inhibiting bacterial adhesion and biofilm formation.[45]

In addition, zwitterionic polymers show significant research prospects in inhibiting protein aggregation. Traditionally, scientists have used polyethylene glycol (PEG) to modify proteins to improve their solubility, hydration size, and reduce kidney clearance, thereby extending their circulation time in the body.[46] However, recent research has revealed that it can sometimes trigger allergic reactions and immune responses that can be life-threatening.[47]

However, using zwitterionic polymer can significantly optimize these

problems. By carefully regulating the chemical structure of zwitterionic polymers, we can tailor polymer compounds to meet specific needs, thus achieving more precise protection of proteins.

It has been reported that a zwitterionic polymer has been successfully synthesized, which can inhibit the aggregation of lysozyme and maintain certain enzyme activity under heating conditions. This novel zwitterionic polymer, polysulfobetaine (poly-SPB), shows significant potential in inhibiting lysozyme aggregation.[48,49]

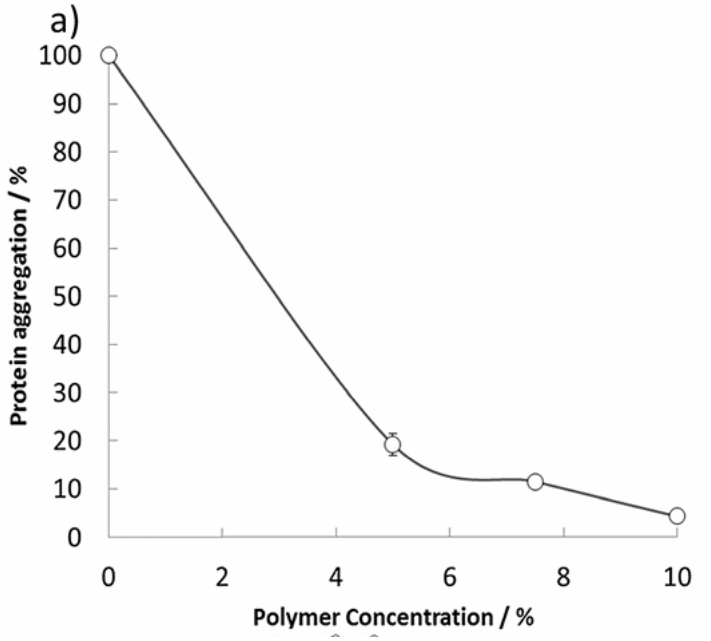


Figure 1.11 (a) Protein aggregation of lysozyme (0.5 mg mL 1) when heated to 90 ℃ for 30 minutes in the presence of poly-SPB at various polymer detected by ThT. [48]

As can be seen from Figure 1.11(a), with the increase of the polymer concentration, the efficiency of the polymer to inhibit protein aggregation is getting better and better. At the concentration of 10% (100mg/mL), almost all proteins do not aggregate even if heated for 30 minutes.

In addition, by comparing other kinds of reagents at the same concentration (50mg/mL), it was found that this new polymer has better efficiency. Figure 1.11(b)

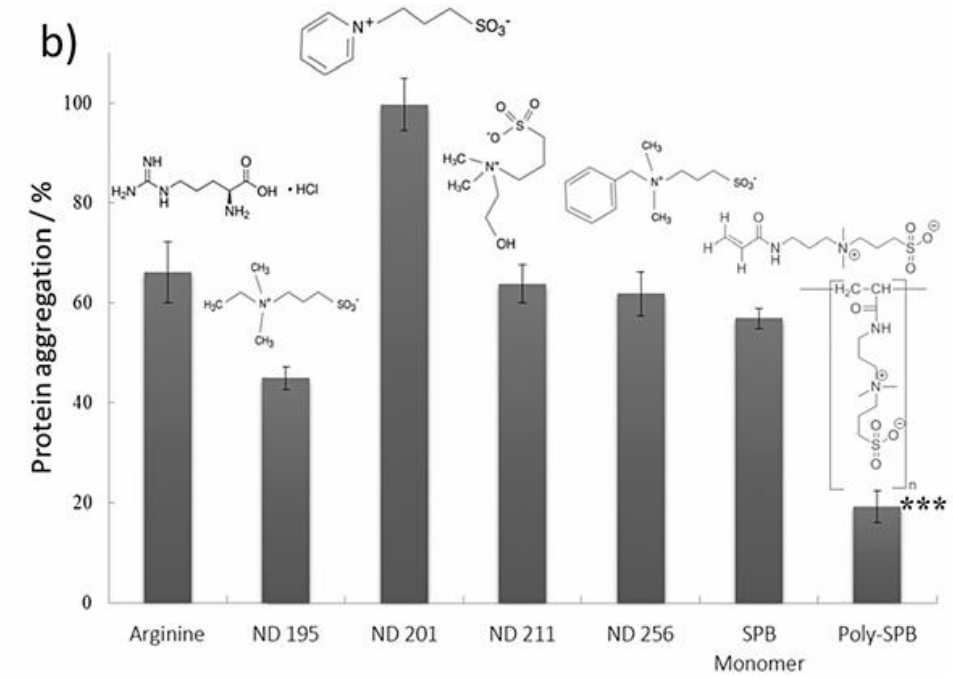


Figure 1.11 (b) In the presence of various reagents (5% w/v) detected by ThT. [48]

More importantly, through the detection of the remaining enzyme activity, it was found that at a higher concentration (150mg/mL) of Poly-SPB, the protein can almost maintain about 90% activity.

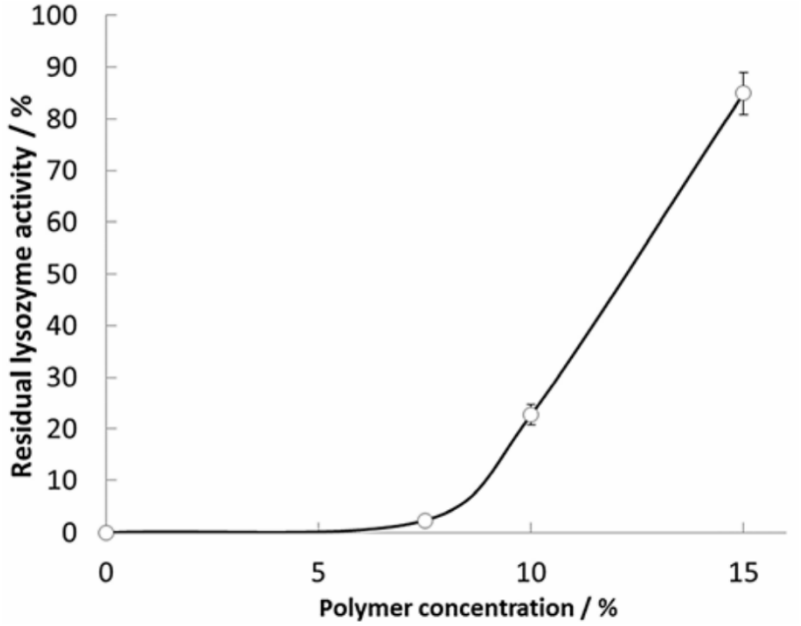


Figure 1.12 Enzymatic activity of lysozyme after treatment at 90 ℃ in the presence of poly-SPB. [48]

During the experiment, special attention was paid to the behavior of poly (SPB) under extreme conditions. The results show that poly-SPB can effectively prevent the aggregation of lysozyme, even in harsh environments with extremely high temperatures and prolonged heating. As far as we know, this is the first time that zwitterionic polymers have been applied to inhibit protein aggregation. This breakthrough not only broadens the application range of polymer materials, but also provides new ideas and methods for solving the problem of protein aggregation.

But at the same time, I also found that a shortcoming of this polymer is that a relatively high concentration is required to achieve a relatively ideal efficiency of inhibiting protein aggregation.

1.4 Objective of study

In my research, I plan to synthesize a kind of polyampholyte, which is a polymer whose structure is highly like that of a zwitterionic polymer. This polyampholyte electrolyte was designed to explore its potential applications in regulating protein behavior. Especially tried to increase the efficacy at lower concentration by the specific polyampholytes.

In the experimental phase, I plan to mix this polyampholyte with a protein solution. By precisely controlling the polymer to protein ratio, I expect to be able to observe the interaction between the two. Subsequently, I will use heat to induce protein aggregation. The key to this step is to study how proteins behave under physiological or pathological conditions by using temperature changes to simulate the environmental changes they may experience.

After the heat treatment, I will carefully examine the protein sample, paying special attention to whether it has undergone denaturation. Protein denaturation is a complex process involving the destruction of its three-dimensional structure and loss of function. Therefore, the detection of protein denaturation is not only the verification of experimental results, but also a direct evaluation of the effect of polyampholyte.

My research hypothesis is that the aggregation process of proteins can be effectively inhibited in the presence of polyampholyte. This inhibitory effect may result from electrostatic interactions between the polymer and the protein, steric effects, or other yet unspecified mechanisms. Through systematic experimental design and data analysis, I hope to reveal the nature of this inhibition and provide theoretical basis and experimental support for the development of more effective protein stabilizers in the future.

Chapter 2

Protein protection by hydrophobic modified PLL-SA

2.1 Introduction

Polyampholytes polymer, as a kind of unique zwitterionic polymer, its structure and properties show remarkable uniqueness in the field of materials science. These polymers consist of a mixture of charged monomer subunits, whose chemical structure units are cleverly integrated with ionizing groups, giving them excellent ionic conductivity. Compared with traditional zwitterionic polymers, polyampholytes polymers show a more complex structure, and this difference in structure directly affects their physical and chemical properties.

In conventional zwitterionic polymers, the anion and cationic groups usually coexist in the same monomer unit, forming an internal charge balance structure.[50,51] Although this design ensures the stability and functionality of the polymer under certain conditions, its performance may be limited in some application scenarios. In contrast, polyampholytes polymers disrupt this internal charge balance by distributing anion and cationic groups across different monomer units, thereby unlocking the potential properties of the polymer to a greater extent (Figure 2.1). [50,51]

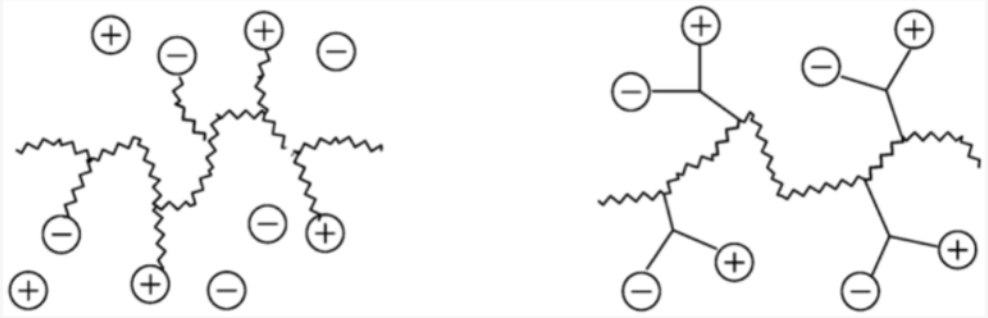


Figure 2.1 The models of polyampholyte (left) and zwitterionic polymers (right).[52]

This structural innovation brings many advantages. Firstly, polyampholytes are more prominent in ionic conductivity. Because the anion and cationic groups are distributed on different monomer units, a more complex charge network is formed inside the polymer. This network structure can conduct ions more efficiently under the action of electric field, thus improving the conductive property of the polymer. Secondly, this structural design also enhances the mechanical properties and thermal stability of the polyampholytes polymer.[53] By dispersing the charge groups, the interaction forces between the polymer molecules are optimized, making the polymer exhibit better stability under mechanical stress and high temperature environments.[53]

In addition, the application prospect of polyampholytes polymers is also broader because of its unique structure. In energy storage, sensors, biomedicine and other fields, polyampholytes polymers show great application potential due to their excellent ionic conductivity and versatility. For example, in batteries and supercapacitors, polyampholytes polymers can be used as efficient electrolyte materials to improve the energy density and cycle life of devices [54]; In biosensors, its excellent ionic conductivity is helpful to improve the sensitivity and response speed of the sensor. [55] In drug delivery systems, the biocompatibility and controlled release properties of polyampholytes polymers provide new solutions for precise drug delivery.[56]

Zwitterionic polymers and polyampholytes polymers, with their unique double charge properties, show significant advantages in the simulation of protein properties.[57] These polymers not only accurately reproduce the charged state of the protein, but also interact more closely with biological systems at the molecular level. This high similarity lays a solid foundation for their wide application in the biomedical field.

What is more striking is that the modifiable properties of amphoteric polymers provide unlimited possibilities for the expansion of their application range. By introducing environmentally responsive groups or structural units, polymers can be given properties similar to those of the current popular environmentally responsive polymers. For example, changes

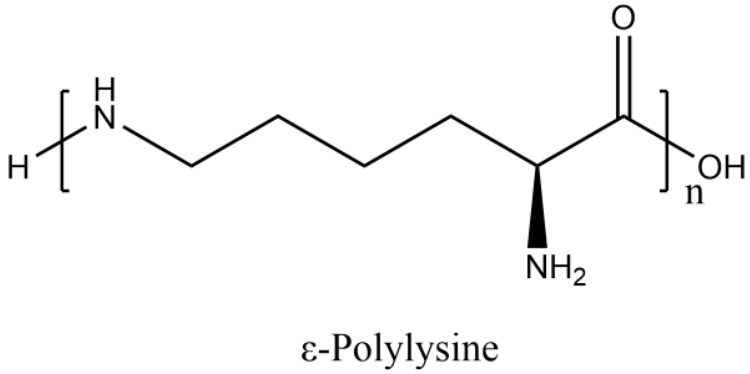
in temperature, pH, or ionic strength can trigger morphological or functional transformations in polymers that enable advanced functions such as smart drug delivery or biosensing. This flexibility not only enhances the adaptability of polymers for specific applications, but also provides new ideas for developing a new generation of biomaterials and medical devices.

As mentioned earlier, the key to proteins' diverse functions lies in their unique spatial folding structure. Whether in vivo or in vitro, the root cause of protein denaturation and aggregation lies in the destruction of its three-dimensional structure. This destruction makes the original hidden in the protein molecules inside the group exposed, the mutual attraction between the group and then triggered the protein aggregation phenomenon.

As a natural compound, α-amino acid not only plays a vital role in living organisms, but also its unique zwitterionic structure gives it a unique advantage in polymer synthesis. As the building blocks of proteins, amino acids can not only act as side chains of zwitterionic polymers, but also undergo a variety of chemical modifications through their rich functional groups. The synthesis of polyampholytes polymers using amino acid polymers as the backbone has many advantages. First, there are a wide range of natural sources of amino acids, which provide a solid foundation for mass production. Secondly, due to the excellent biocompatibility of amino acids themselves, polyampholytes polymers based on their synthesis can effectively avoid immune reactions, which is particularly important for their applications in the biomedical field. In addition, amino acid polymers are easy to carry out subsequent modifications, which provides the possibility to customize the design of polymer materials with specific functions.

ε-Polylysine (Figure 2.2) is a homopolymer formed from L-lysine via an isopeptide bond between its ε-amino and γ-carboxyl groups. This polymer presents a linear arrangement in structure and is composed of 25-30 lysine monomers, giving it unique physical and chemical properties.[58] It is important to note that although PLL can be obtained by chemical synthesis, biogenic PLL is naturally produced by the fermentation of Streptomyces albulus.[104]

The preparation process of bioderived PLL involves complex microbial metabolic pathways that ensure a high purity and specific molecular weight distribution of the polymer. Compared to chemical synthesis, biosynthesized PLL is more homogeneous in structure and does not contain any possible by-products or impurities, which makes it a significant advantage in applications in food, medicine and biomaterials.



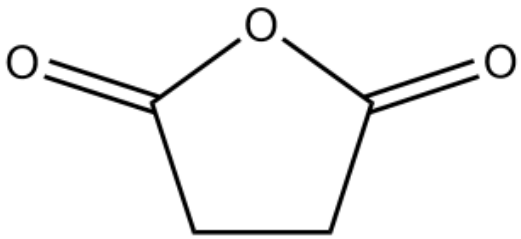
ε-Polylysine

Figure 2.2 The chemical structure of the PLL.

ε-polylysine, not only performs well in safety, but also shows significant advantages in the field of food preservation and antibacterial. First, its safety is based on extensive ADMET studies, which confirm that ε-polylysine is non-toxic and harmless during absorption, distribution, metabolism, and excretion in the human body, and does not trigger any immune response.[59] This safety has led to ε-polylysine being approved as a natural food preservative in countries such as the United States and Japan with GRAS number No. 000135, further demonstrating its value in the food industry.[60] In terms of technical properties, ε-polylysine has outstanding modification potential. It can be combined with other monomers, chemical modification, at the same time, this substance also has good thermal stability. This thermal stability allows ε-polylysine to be processed at more severe temperature conditions, which is an important advantage for the food industry as it can be adapted to a variety of complex production environments. In addition, the antimicrobial spectrum of ε-polylysine is extremely broad, which makes it significantly effective in inhibiting the growth of a variety of bacteria. Whether it is gram-negative bacteria or Gram-positive bacteria, ε-polylysine

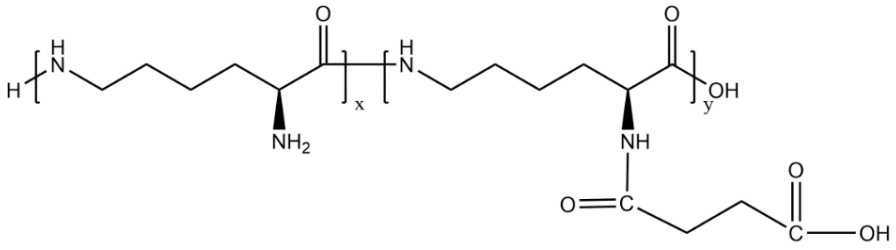
can show strong inhibition ability, which provides a more comprehensive protection for food preservation.[61]

The strategy of reacting Succinic Anhydride (SA) with ε-Poly-L-lysine (ε-PLL) not only successfully synthesized polyamphotic electrolyte (PLL-SA), but also provided a new way to precisely regulate its surface charge characteristics. As shown in Figure 2.3, the modification with succinic anhydride involves the reaction of the amino group of ε-PLL with SA to form the succinylated ε-PLL, PLL-SA (Figure 2.4). This chemical modification not only changes the charge distribution of ε-PLL, but also significantly enhances its solubility and biocompatibility.



Succinic anhydride

Figure 2.3 The chemical structure of the SA.



PLL-SA

Figure 2.4 The chemical structure of the PLL-SA.

The surface charge characteristics of PLL-SA can be precisely regulated by adjusting the amount of SA added. Specifically, with the increase of the molar ratio of SA, the negative charge density of PLL-SA gradually increases, while the positive charge density correspondingly decreases.

This change in charge characteristics makes PLL-SA exhibit different charge behaviors in different pH environments, thus broadening its application potential in biomedicine, materials science and other fields. For example, in drug delivery systems, by regulating the charge properties of PLL-SA, it is possible to optimize its interaction with target cells and improve the targeting and efficacy of drugs.

Previous studies have concluded that the introduction of hydrophobic monomers into polymer systems can significantly improve their ability to inhibit protein aggregation.[62] The effectiveness of this strategy is mainly due to the specific interaction formed between the hydrophobic monomer and the protein, which can interfere with the aggregation tendency of the protein molecules, thereby maintaining the dispersed state of the protein.

Furthermore, there is a close relationship between surface charge distribution and hydrophobicity.[63] Increased hydrophobicity usually results in a redistribution of the surface charge of the polymer, a change that has profound implications for the stability of the particles.[63] Specifically, the addition of hydrophobic monomers may change the charge density and distribution on the polymer surface, thus affecting the electrostatic repulsion between particles. This change in electrostatic repulsion is essential to prevent the agglomeration of protein particles. In addition, hydrophobic monomers may also regulate the interaction between proteins and polymers by changing the hydrophilic and hydrophobic equilibrium of polymers.[64] This adjustment of balance allows the polymer to more effectively enclose and disperse proteins, further enhancing its ability to inhibit protein aggregation.

During the experiment, to further improve the hydrophobic performance of ε-Poly-L-lysine-Succinic Anhydride (PLL-SA), I carefully introduced dodecyl succinic anhydride (DDSA), the specific structure of which is shown in Figure 2.5. Compared with traditional succinic anhydride (SA), DDSA shows significant advantages in chemical structure. Its carbon chain length is more extended than that of SA, which gives DDSA more excellent hydrophobic properties. Moreover, and more

importantly, I found that not only did it perform well in enhancing hydrophobicity, but the addition of DDSA did not introduce new compounds. This strategy effectively avoids the potential factors that may interfere with the experimental results and ensures the purity and reliability of the experimental data.

Therefore, I decided to incorporate DDSA as a key monomer in the synthesis of PLL-SA. With this innovative material design, I expect to be able to take advantage of DDSA's unique structural properties to significantly improve PLL-SA's hydrophobic properties.

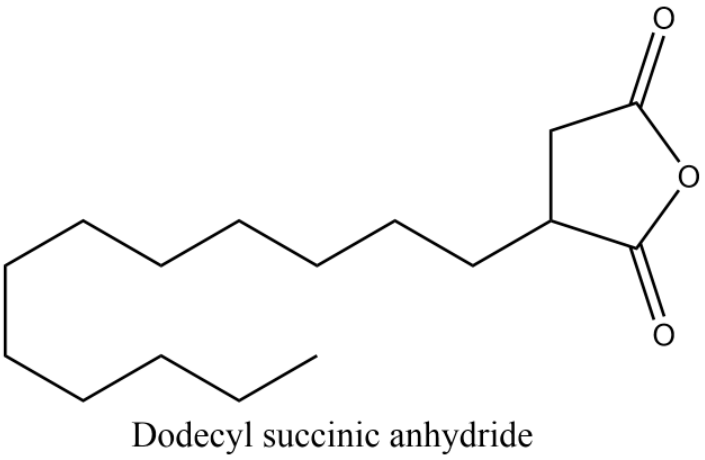


Figure 2.5 The chemical structure of the DDSA.

2.2 Materials

25% (w/w) ε-Polylysine (PLL) (molecular weight 4000) aqueous solution was purchased from JNC CORPORATION (Tokyo, Japan). SA (Succinic Anhydride) was purchased from NACALAI TESQUE, INC (Kyoto, Japan). DDSA (Dodecylsuccinic Anhydride) was purchased from TOKYO CHEMICAL INDUSTRY (Tokyo, Japan). Dulbecco PBS (-) powder was purchased from NISSUI PHARMACEUTICAL CO.,LTD (Tokyo, Japan). Lysozyme from chicken egg white, Micrococcus lysodeikticus and LDH (Lactate Dehydrogenase) from rabbit muscle were purchased from Sigma-Aldrich (St. Louis, MO)

2.3 Synthesis of Polymer

2.3.1 Synthesis of PLL-SA

At a temperature of 50℃, I mixed 10 mL of polylactic acid (PLL) with 1.2675g of succinic anhydride (SA) and heated it for 2 hours. Through this process, I successfully neutralized 65% of the amino group in PLL, thus preparing PLL-SA (65), as shown in Figure 2.6 a.

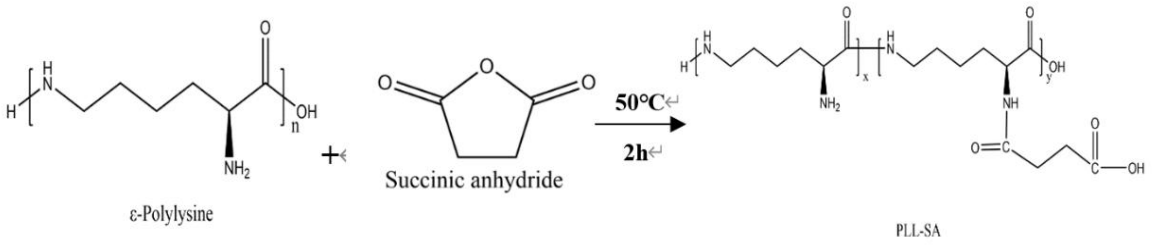


Figure 2.6 a Synthesis of ε-Polylysine with SA.

2.3.2 Synthesis of PLL-DDSA-SA

As mentioned earlier, the introduction of hydrophobic monomers into the polymer significantly improves its efficiency in inhibiting protein aggregation. The surface charge distribution is regulated by hydrophobicity, which affects the stability of the particles. The main difference between DDSA and SA is that DDSA contains a long chain of 12 alkyl groups. And no new impurities will be introduced, so I chose DDSA as the monomer in the experiment. During the experiment, PLL-SA (65%) was first synthesized, and then different proportions of DDSA were gradually added (2%, 5%, 8%, 10%, respectively, according to the quality of DDSA). Specifically, the mass of DDSA is 0.10452g (corresponding 2%), 0.2617g (corresponding 5%), 0.42g (corresponding 8%) and 0.5234g (corresponding 10%), respectively. These experiments are shown in Figure 2.6 b.

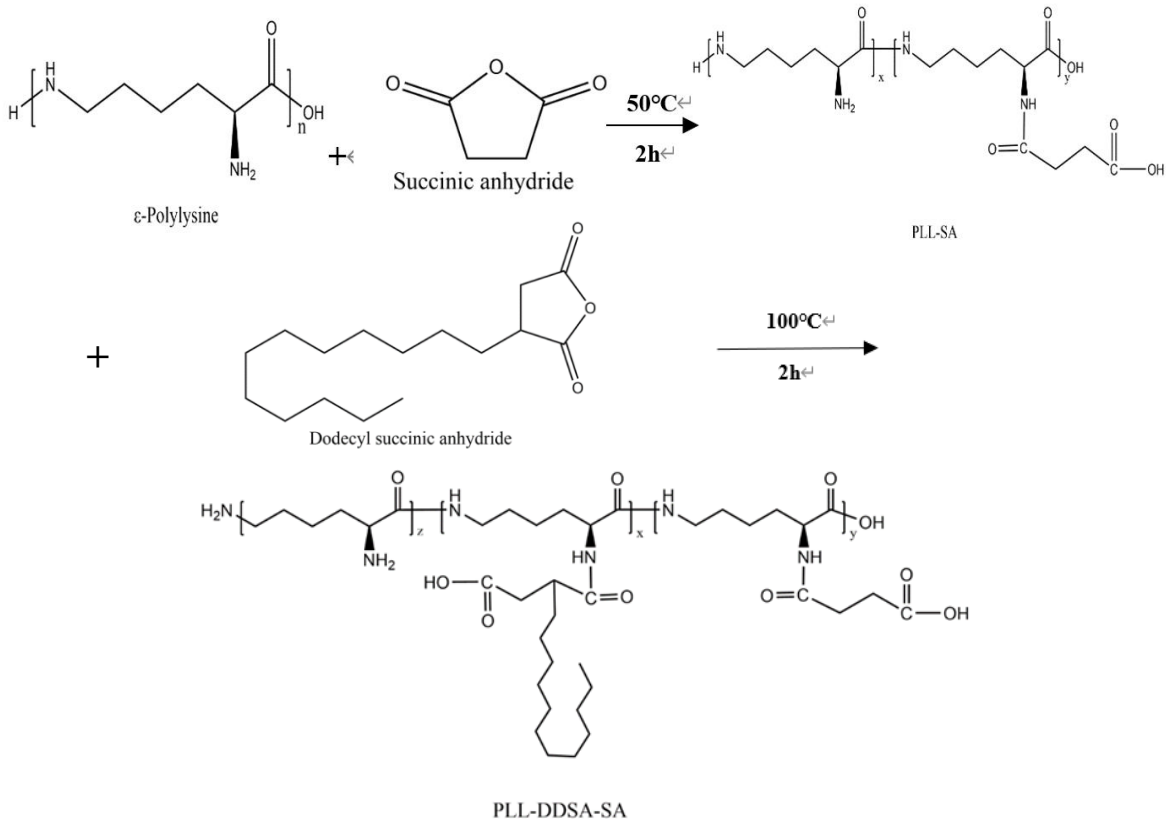


Figure 2.6 b Synthesis of ε-Polylysine with DDSA

2.4 Characterization of the polymers and protein

All polymers were thoroughly characterized by ^{1}H nuclear magnetic resonance spectroscopy (NMR) for in-depth analysis of their chemical structure and composition to ensure that the resulting polymer met the expected requirements. After the sample was dried, 100mg of polymer was dissolved in 700μL D_2O. The chemical structure changes of lysozyme before and after heating were detected by nuclear magnetic resonance technology.

The chemical structure of all polymers was analyzed in detail using a 400 MHz Bruker AVANCE III spectrometer, and the NMR data was precisely processed and analyzed using Topspin 3.5 software.

2.5 Inhibition of the protein aggregation

In the experimental design, I selected lysozyme as a model protein to explore the potential of polylysine-succinic acid (PLL-SA) and polylysine-dissuccinic acid-succinic acid (PLL-DDSA-SA) to inhibit protein aggregation. Lysozyme, due to its detailed analysis of primary and tertiary structures, has been widely recognized and is often used as a template protein to evaluate the effect of compounds on inhibiting protein aggregation. By using lysozyme, I was able to more accurately evaluate the inhibitory effects of PLL-SA and PLL-DDSA-SA under different conditions, providing strong support for understanding the mechanism of action of these compounds.

During the experiment, I will evaluate the inhibition effect of PLL-SA and PLL-DDSA-SA on lysozyme aggregation through a series of rigorous experimental steps. These experimental methods will provide us with quantitative and qualitative data on the inhibition effect of the compounds, and thus reveal the specific mechanisms of their inhibition of protein aggregation

2.5.1 Dynamic Light Scattering

I learned that protein aggregation leads to a significant increase in particle size. Therefore, through Dynamic Light Scattering (DLS) technology, I can not only accurately measure the size of protein particles, but also analyze whether the polymer could inhibit protein aggregation from a physical level.

I used the Zetasizer 300 system (manufactured by Malvern Instruments in Worcestershire, UK) to determine the hydrodynamic diameters of proteins and polymers. The system measures at a scattering Angle of 173°, ensuring the accuracy and reliability of the data.

2.5.2 Residual enzyme activity

During the study, I used a PBS buffer with pH 7.4 to dissolve lysozyme and polymer. The concentration of lysozyme is set at 0.2 mg/ml, while the concentration of polymer is 10 mg/ml. Equal parts of lysozyme solution and polymer solution (0.5 ml each) were mixed and heated in a water bath at 90 ° C for 30 minutes. I then thoroughly mixed 2 ml of Micrococcus lysophilus solution at 0.25 mg/ml (also prepared using PBS buffer) with 100 μL of lysozyme-polymer mixture in a quartz test tube.

To evaluate lysozyme activity, I used the Jasco V-770 spectrophotometer (from Tokyo, Japan) to monitor the absorbance of the above solution at room temperature in real time at 600 nm wavelength, from 0 minutes to 5 minutes. By recording the change in absorbance over time, I can infer the change in lysozyme activity. The slope of the curve directly reflects the residual degree of lysozyme activity.

2.6 Results and discussion

2.6.1 Characterization of the PLL-SA and PLL-DDSA-SA

In this experiment, I successfully prepared a series of polyampholytes electrolytes by using PLL and specific amounts of SA and DDSA. Specifically, II synthesized PL-SA (65%) and PL-DDSA (2%, 5%, 8%, 10%) -SA (65%). In order to verify the chemical structure and composition of these polymers, I performed in-depth analysis using hydrogen NMR spectroscopy. The analysis results are shown in Figure 2.7 (a-e).

It can be clearly observed from the figure that the chemical shift of hydrogen atoms in each different environment is clearly reflected. This finding strongly supports the inference that I have initially synthesized the target compound.

Specifically, Figure 2.7 (a) shows the spectrum of PLL-SA (65%), while Figure 2.7 (b), (c), (d), and (e) correspond to the spectrum of PLL-DDSA (2%, 5%, 8%, 10%) -SA (65%), respectively. These spectral data not only provide us with key information about the structure of the compound, but also further confirm the validity of the experimental design.

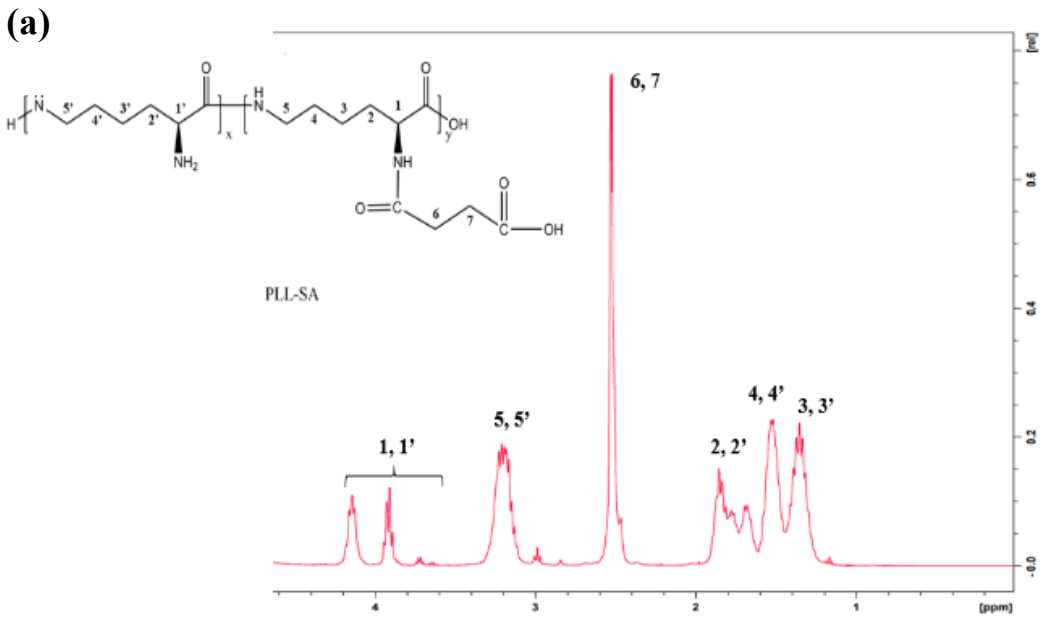


Figure 2.7 (a) The ^{1}H-NMR spectrum of the PLL-SA (65%) in D_2O.

(b)

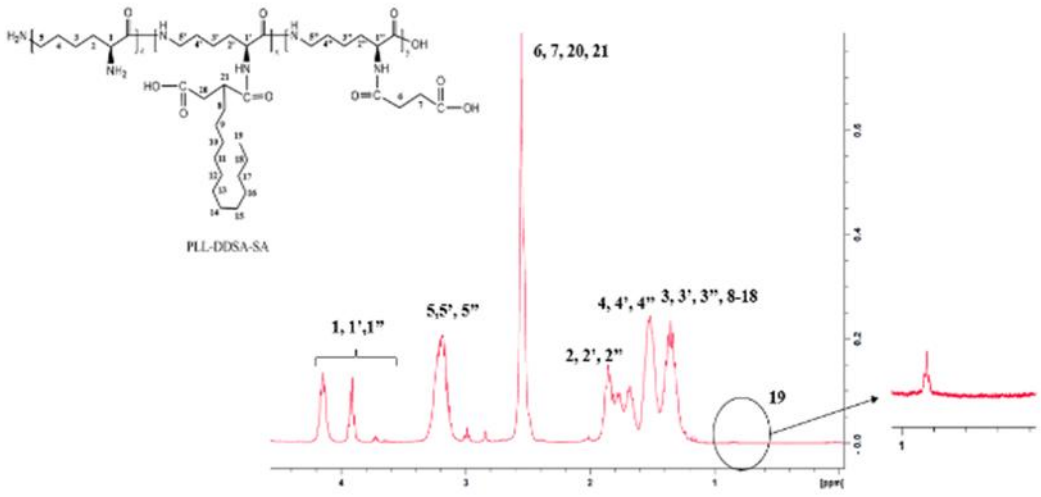


Figure 2.7 (b) The ^{1}H-NMR spectrum of the PLL-DDSA (2%)-SA (65%) in D2O.

(c)

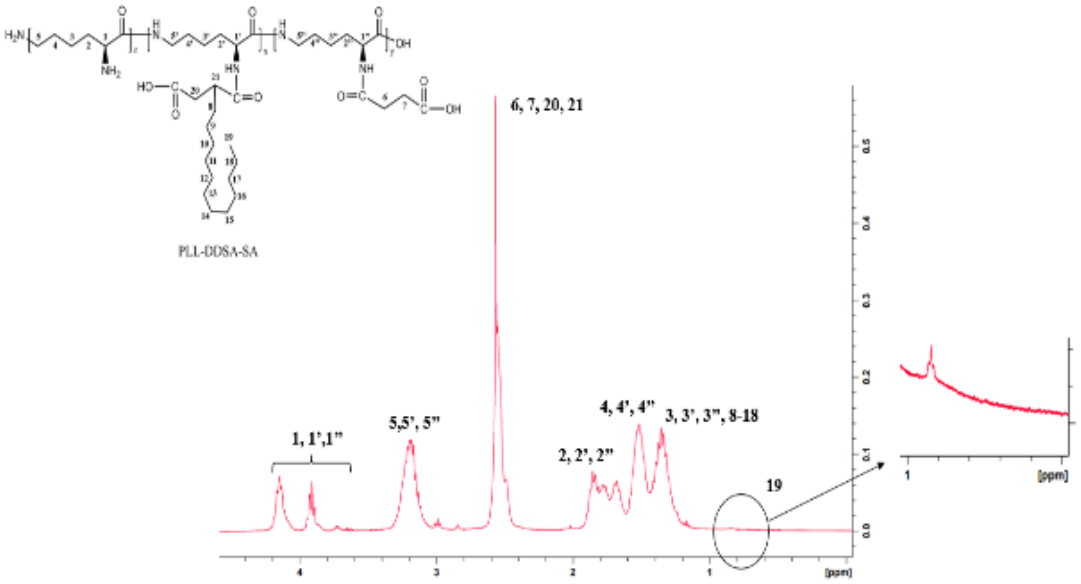


Figure 2.7 (c) The ^{1}H-NMR spectrum of the PLL-DDSA (5%)-SA (65%) in D2O.

(d)

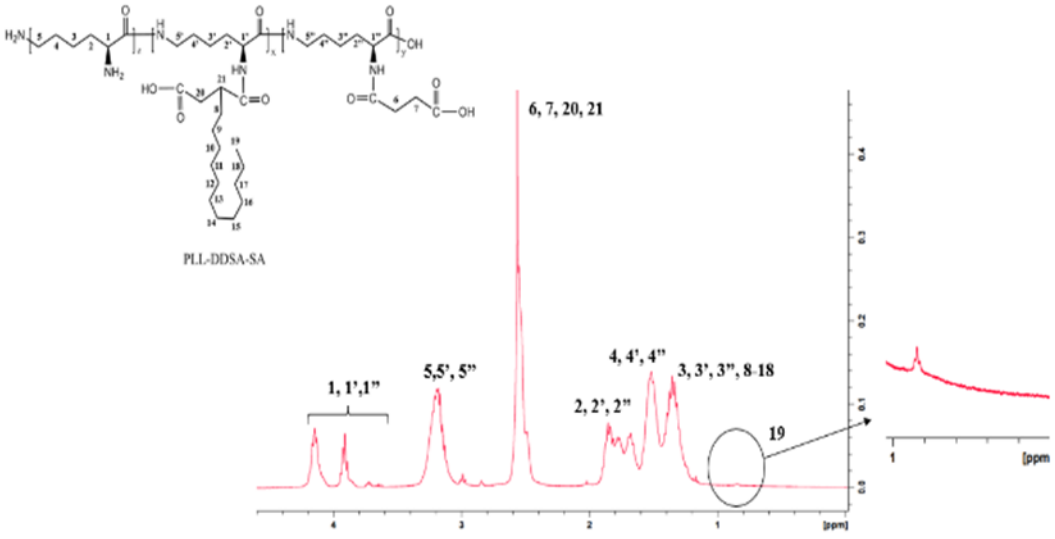


Figure 2.7 (d) The ^{1}H-NMR spectrum of the PLL-DDSA (8%)-SA (65%) in D2O.

(e)

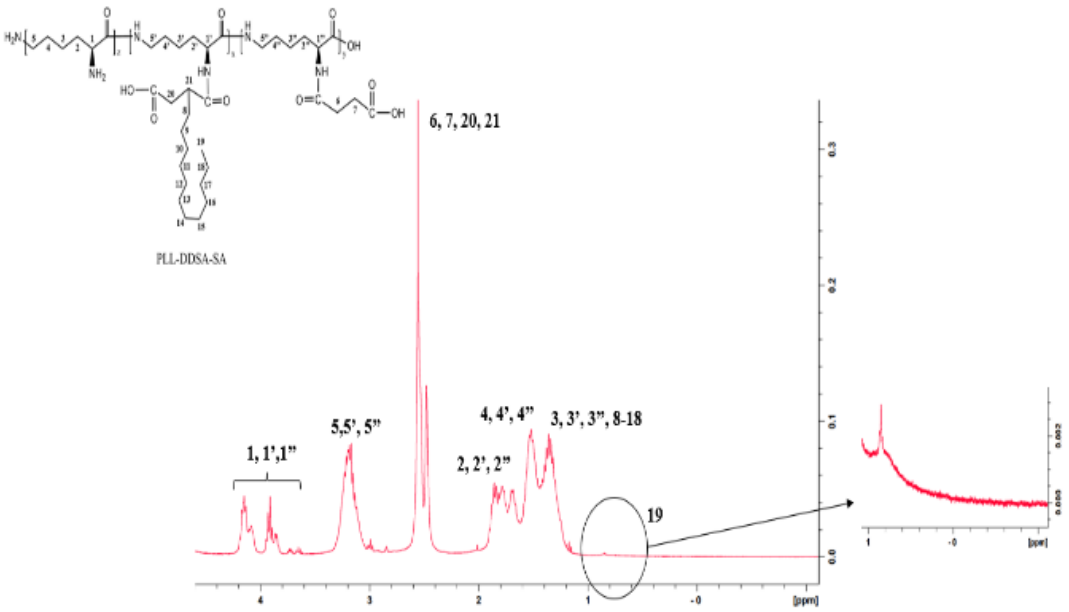
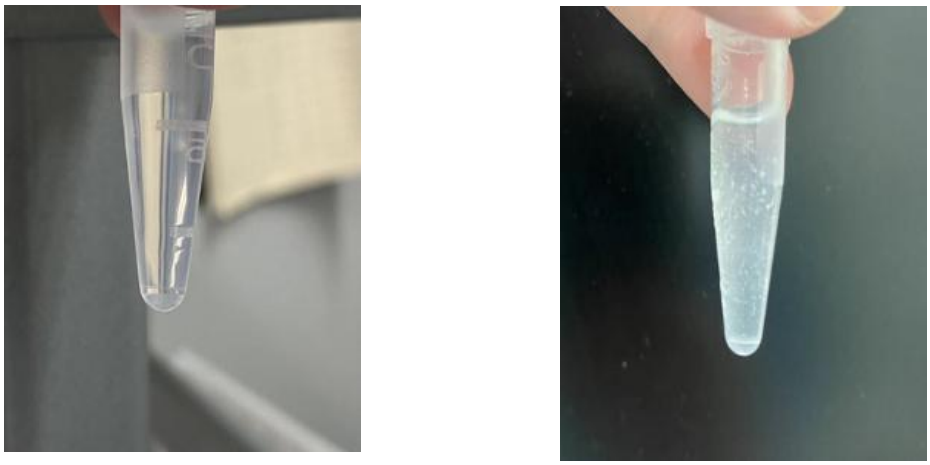


Figure 2.7 (e) The ^{1}H-NMR spectrum of the PLL-DDSA (10%)-SA (65%) in D2O.

2.6.2 The appearances of the lysozyme solution in comparison before and after heating.

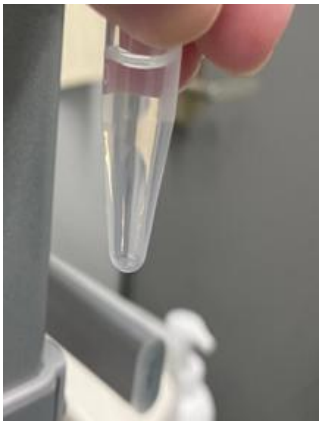
From the figures below (Fig. 2.8 a, b), it can be clearly observed that the heated protein solution (90℃, 30 minutes) presents a significant white precipitation, while the unheated natural protein solution remains clear and transparent in the PBS buffer (pH 7.4).



(a) (b)

Figure 2.8 Only lysozyme before (a) and after (b) heating 30 mins.

Next, PLL-SA (65%) (20 mg/ml) and PL-DDSA (2%, 5%, 8%, 10%) - SA (65%) (20 mg/ml) were mixed with protein (0.4 mg/ml) at different concentrations, respectively, and observed after heating for 30 minutes. It can be clearly seen from Figure 2.9 (a, b, c, d, e) that after 30 minutes of heating treatment, no white precipitate appeared in all mixtures. This phenomenon indicates that the polymer can effectively inhibit the aggregation of proteins to a certain extent. Therefore, I tentatively conclude that this polymer has the potential to inhibit protein aggregation.



(e) PLL-SA (65%)

(d) PLL-DDSA (2%)-SA (65%)

(c) PLL-DDSA (5%)-SA (65%)

(b) PLL-DDSA (8%)-SA (65%)

(a) PLL-DDSA (10%)-SA (65%)

Figure 2.9 The pictures of the PLL-SA and PLL-DDSA-SA mixed with lysozyme after heating 30 mins.

However, I observed that when the protein was mixed with PL-DDSA-SA and heated, the solution became somewhat cloudy compared to the solution without DDSA added. Notably, this phenomenon is not caused by protein aggregation, but by the introduction of DDSA that enhances the hydrophobicity of the polymer, which may lead to the formation of polymer micelles.

To take a deeper look at the protein aggregation in each solution, I used microscopy techniques for detailed analysis. During the experiment, I selected a high-performance BZ-X800 microscope (Keyence Co., Ltd., Osaka, Japan) to ensure the best possible results for each solution. All experimental results have been organized and shown in Figures 2.10 and 2.11.

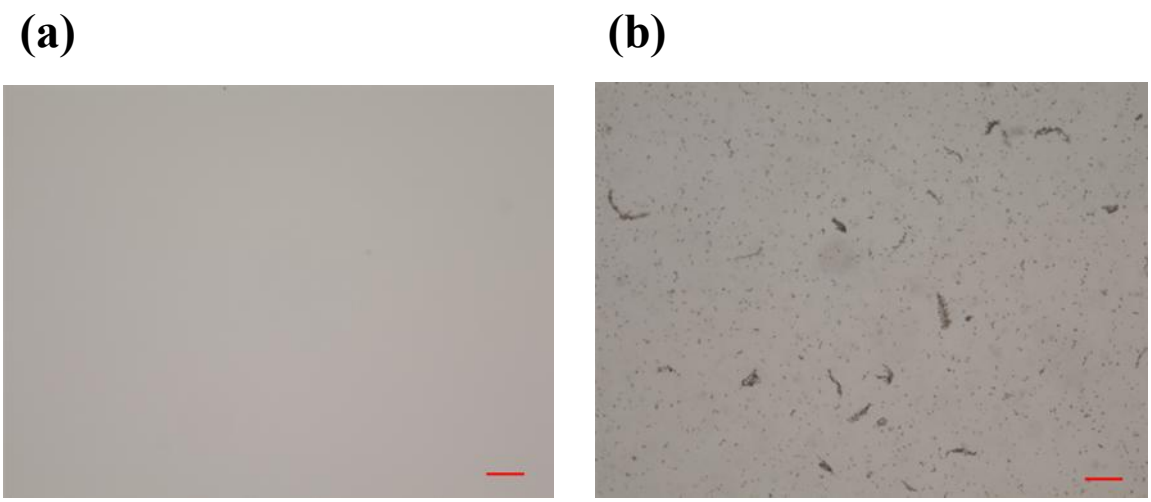


Figure 2.10 Only lysozyme before (a) and after (b) heating 30 mins. Scale bar = 100 µm.

(e) PLL-SA (65%)

(d) PLL-DDSA (2%)-SA (65%)

(c) PLL-DDSA (5%)-SA (65%)

(b) PLL-DDSA (8%)-SA (65%)

(a) PLL-DDSA (10%)-SA (65%)

Figure 2.11 a, b, c, d, e The pictures of the PLL-SA and PLL-DDSA-SA mixed with lysozyme after heating 30 mins. Scale bar = 100 μm.

Under the microscope, I was able to reveal the effect of heating on protein behavior. As shown in Figure 2.10(b), the heat-treated proteins showed aggregation of different sizes, while the unheated proteins maintained their original state and did not show aggregation (Figure 2.10(a)). More interestingly, when the heated proteins were mixed with the polymer, no aggregation was observed. This result strongly suggests that the polymer plays a significant role in inhibiting protein aggregation (Figure 2.11(a), (b), (c), (d), (e)).

2.6.3 Dynamic Light Scattering

Subsequently, I tested the particle size of the protein under heating and without heating (natural state), and the results were shown in Figure 2.12. In the experiment, the protein concentration was set at 0.4 mg/ml, the heating process lasted for 30 minutes, and the temperature was maintained at 90 ° C.

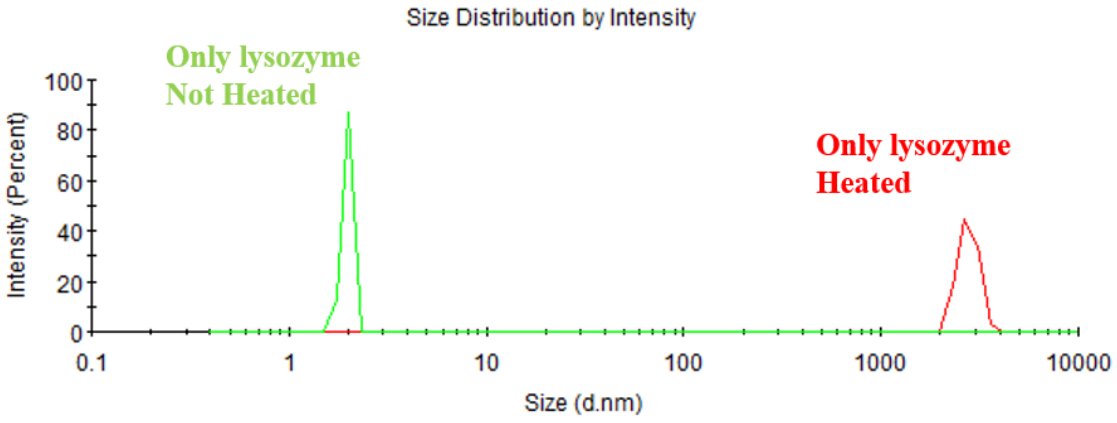


Figure 2.12 The size of the protein, heating (red) and not heating (green).

It can be clearly observed from the figure that the particle size of the protein increases significantly after heating, which indicates that the protein aggregation occurs during the heating process. In contrast, unheated proteins appear as extremely tiny particles.

Further, a mixture of protein (0.5ml at a concentration of 0.4mg/ml) and polymer (0.5ml at a concentration of 20mg/ml) was heated for 30 minutes. Figure 2.13 shows the particle size change of PLL-SA and protein mixture after heating.

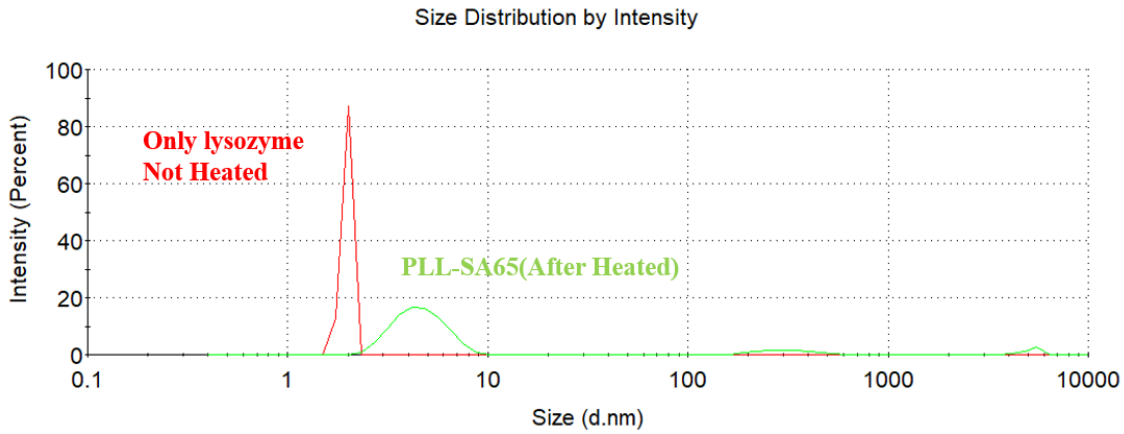


Figure 2.13 The size of the mixture, after heating 30 mins.

When the protein was mixed with PLL-SA, its behavior was highly consistent with that of the unheated protein. In particular, the mixture does not exhibit any significant aggregation, which is fully reflected in the maintenance of particle size. The experimental data showed that the particle size of the mixed protein remained in a very small range, which was consistent with the particle size distribution of the unheated protein.

Next, Figure 2.14 shows the size change of PLL-DDSA-SA mixture after heating with protein for 30 minutes.

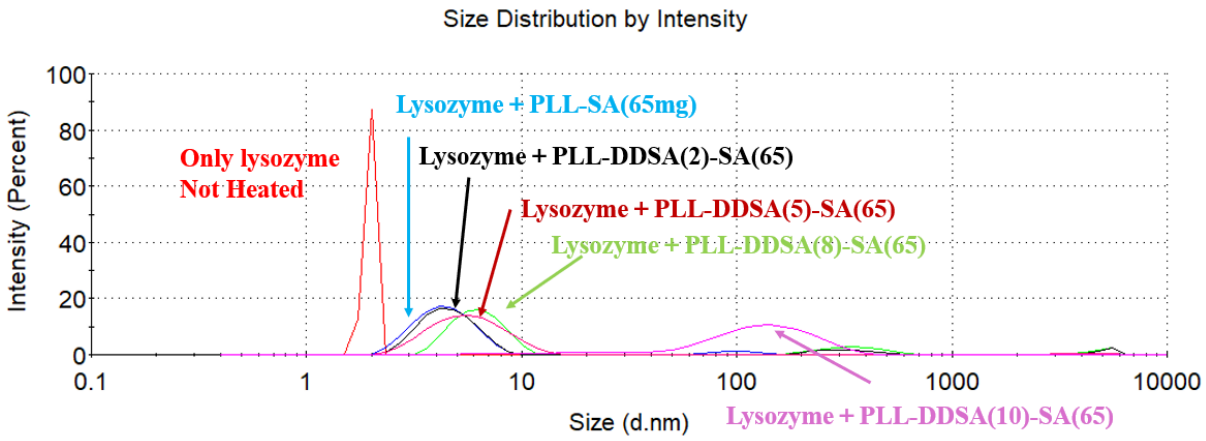


Figure 2.14 The size of the mixture, after heating 30 mins.

It can be clearly observed from the figure that the particle size of the mixture shows an increasing trend with the gradual increase of DDSA percentage. However, it is worth noting that even at the highest DDSA content, the particle size of the mixture remained below the heated protein particle size. This phenomenon indicates that although DDSA has a certain effect on particle size, its effect is not beyond the significant change of protein particle size after heating treatment. (Figure 2.14)

To verify whether large particles are protein aggregates, I conducted the following comparative tests. First, I measured the particle size of the protein and PLL-DDSA-SA mixture respectively before heating. The measurement results are shown in Figure 2.15.

In addition, for further proof, I also tested the particle size of the PL-DDSA-SA polymer with unmixed proteins only in the unheated state. The result is shown in Figure 2.16.

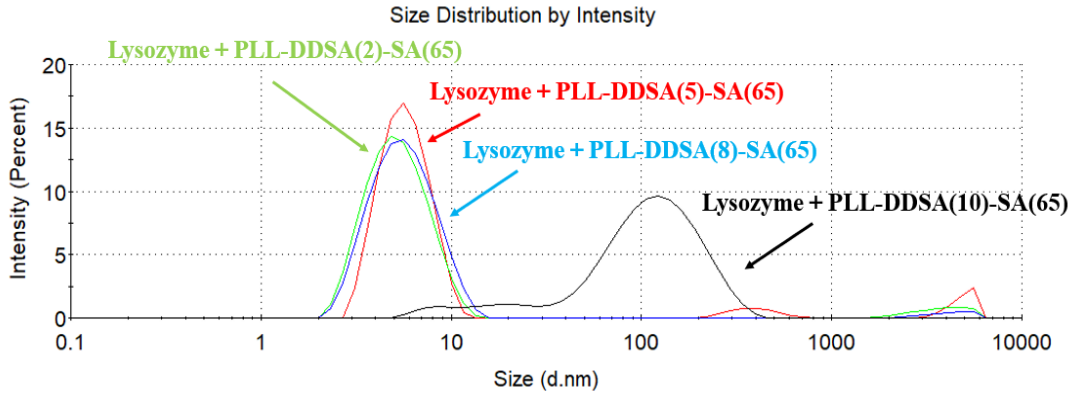


Figure 2.15 The particles' size of the mixtures of the protein and the PLL-DDSA-SA before heating

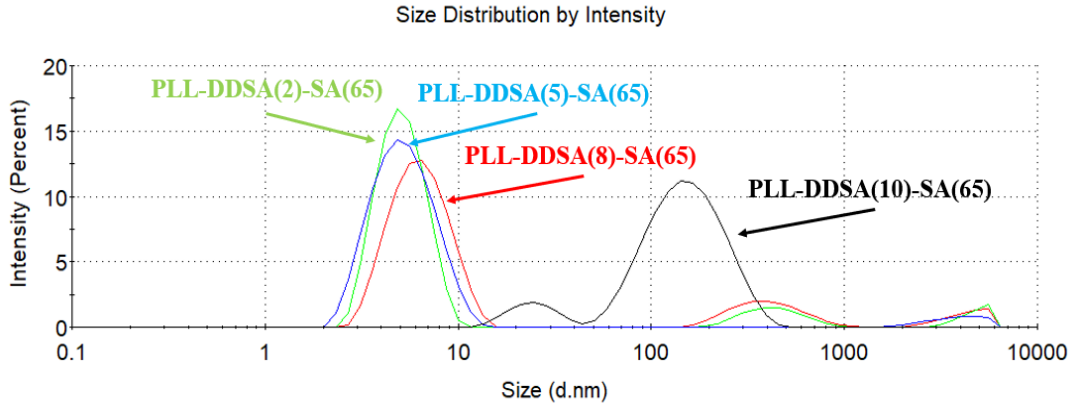


Figure 2.16 The particles' size of the PLL-DDSA-SA before heating

It can be clearly observed from the figure that even in the unheated state, the particle size of the mixture shows a tendency to gradually increase with the increase of DDSA percentage. I have measured the particle size of unheated proteins, and the results show that the particle size is very small without heating. Therefore, Ican infer that the large size of the mixture is mainly due to the hydrophobicity of DDSA. With the increase of DDSA content, the hydrophobicity of the polymer gradually increases, which makes the polymer more prone to aggregation in solution and may form a micellar structure. In addition, by comparing the data in the figure, I can also find that the change rule of material particle size before and after mixing protein has hardly changed. This further confirms our hypothesis that the change in particle size is mainly caused by the micelles formed by the polymer, rather than the proteins themselves.

2.6.4 Residual enzymatic activity

Next, I conducted an experiment aimed at exploring whether the lysozyme in the mixed polymer was denatured after heating. As far as we know, when lysozyme is mixed with micrococcus lysoclysticus, the turbidity of the solution is significantly reduced. Micrococcus lysosus is a yellow bacterium that presents a cloudy state after being dissolved in PBS solution. Lysozyme can break down the cell wall of the micrococcus, causing the bacteria to dissolve, thus making the solution clear. In this process, the absorbance of the sample will be significantly reduced. However, if the lysozyme loses its enzymatic activity, it cannot destroy the cell wall of the lysococcus, and the absorbance of the solution will remain unchanged.

First, I examined the enzyme activity in the unheated state to see if the intervention of the polymer influenced the lysozyme itself. The result is shown in the figure 2.17.

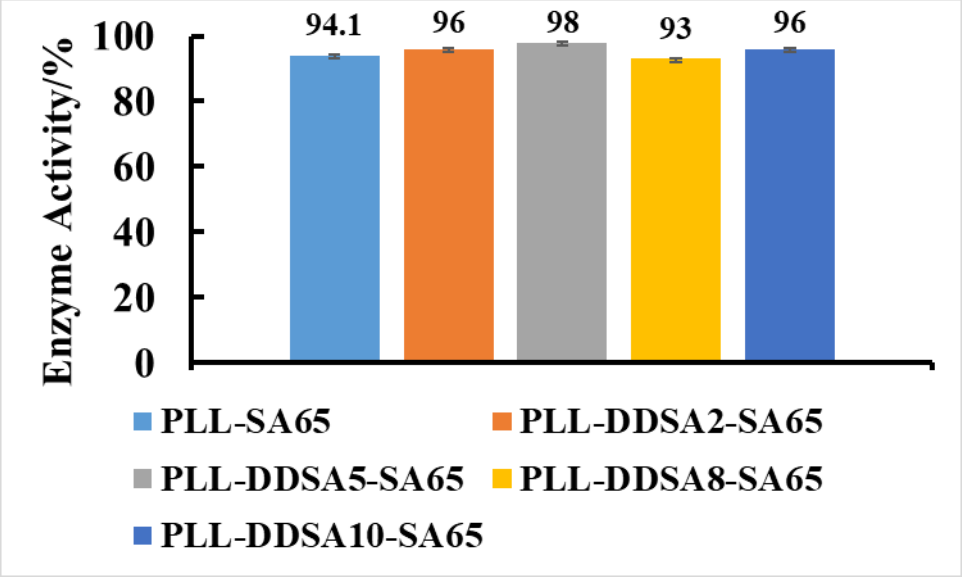


Figure 2.17 The unheated lysozyme activity changes in the presence of the polymer.

It can be seen from the figure that the presence of polymer hardly has a great impact on the activity of lysozyme

Next, I tested the residual enzyme activity of lysozyme (0.2 mg/ml) before and after heating for 30 minutes without adding polymer. The test results are shown in Figure. 2.18 a, which clearly shows the changes of enzyme activity before and after heating.

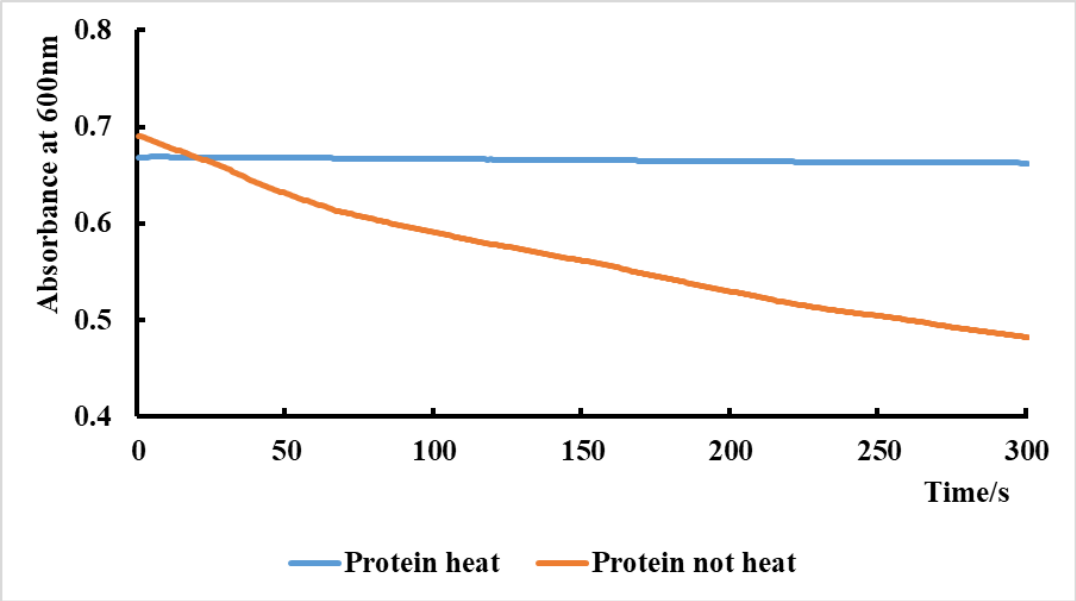


Figure 2.18 a Change in absorbance of Micrococcus lysodeikticus suspension at 600 nm in the presence lysozyme before and after heating.

In the absence of heat, just by examining the proteins, I obtained a curve of continuous decline. The slope of this curve accurately reflects the level of residual lysozyme activity. However, when the lysozyme underwent heating treatment, the absorbance did not change, which clearly indicates that the lysozyme has lost its biological activity.

Next, the residual enzyme activity of lysozyme after mixing the polymer was detected. After 30 minutes of heating treatment, the experimental results are shown in Figure 2.18 b

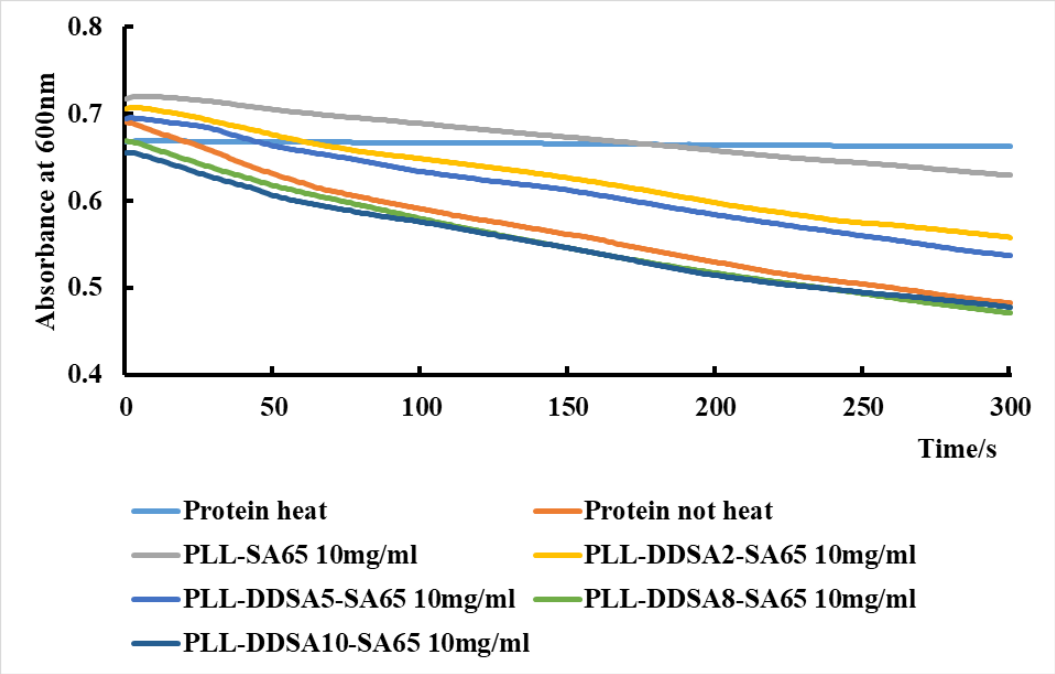


Figure 2.18 b Change in absorbance of Micrococcus lysodeikticus suspension at 600 nm in the presence lysozyme and PLL-SA (65%), PLL-DDSA (2%)-SA (65%), PLL-DDSA (5%)-SA (65%), PLL-DDSA (8%)-SA (65%) and PLL-DDSA (10%)-SA (65%).

It can be clearly observed from the above data that after mixing with the polymer and heating for 30 minutes, the absorbance of lysozyme showed a consistent decreasing trend with that of the natural protein. This phenomenon strongly suggests that lysozyme can maintain its biological activity without being significantly affected even in the presence of polymers. In addition, the linear slope increased with the increase of DDSA, which further revealed an important rule: the higher the DDSA content, the more significant the inhibition effect of the polymer on protein aggregation.

For a more intuitive comparison of the results, I used a bar chart to show the efficiency of this polymer in inhibiting proteins, as shown in Figure 2.19. To ensure the accuracy of the data in the experiment, each sample was measured three times, and its average value was taken.

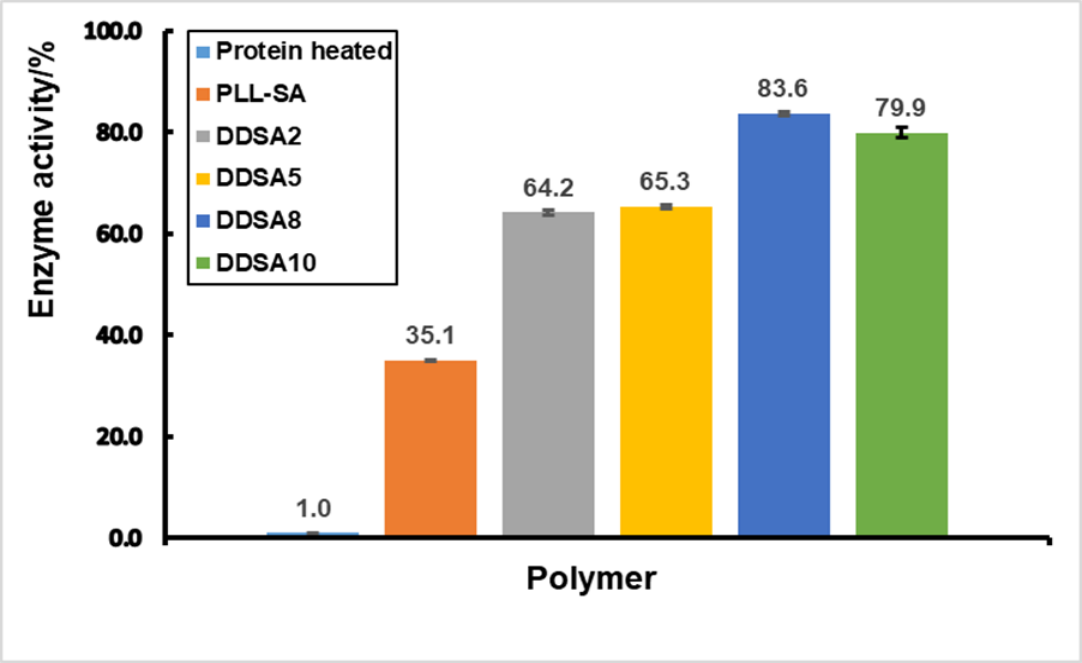


Figure 2.19 Contrast diagram of enzymatic activities of different polymers.

In the experiment, the activity of the protein without the added polymer was almost completely lost after high temperature treatment, however, when the polymer was introduced, especially the polymer containing hydrophobic groups, the lysozyme showed high activity after heating at 90 degrees Celsius for 30 minutes.

The experimental results not only confirmed the protective effect of the polymer on the protein at high temperature, but also revealed that the hydrophobic groups also played a key role in enhancing the stability of the protein. By interacting with the hydrophobic region on the protein surface, the hydrophobic groups form a protective barrier that effectively prevents the denaturation and aggregation of the protein at high temperatures. This interaction not only maintains the three-dimensional structure of the protein, but also maintains its catalytic activity, thus ensuring the functionality of lysozyme under extreme conditions.

2.7 Conclusion

In this part of the study, I synthesized a polyampholytes polymer (PLL-SA and PLL-DDSA-SA) and investigated its inhibitory effect on protein aggregation. First, I synthesized polyampholytes electrolyte (PLL-SA) and enhanced its hydrophobicity by gradually increasing the proportion of DDSA (2%, 5%, 8% and 10%, respectively) to form a series of PLL-DDSA-SA polymers (in which the SA content was fixed at 65%). Subsequently, I characterized these polymers by ^{1}H-NMR, and the results confirmed that the required amounts of SA and DDSA were successfully embedded into the PLL structure.

To evaluate the ability of these polymers to inhibit protein aggregation, I employed dynamic light scattering (DLS) techniques. The results show that lysozyme forms large aggregates when heated alone, and the pre-addition of polymers can completely inhibit this aggregation. In further experiments, I examined the residual enzyme activity of Micrococcus lysosomes before and after heating and after adding the polymer. The results showed that the presence of polymer significantly retained enzyme activity, and with the increase of DDSA content, the enzyme activity also showed a significant increase. PLL-DDSA8-SA65 polymers retain more than 80% of the enzyme activity, fully demonstrating the great potential of these polymers. In addition, further studies have explored the protective effect of these polymers against other proteins (lactate dehydrogenase), and the results likewise show that the polymers can provide good protection even at low concentrations.

In summary, I have successfully prepared a series of polyampholytes electrolytes (PLL-DDSA-SA) with high protein protection efficiency. These polymers can still maintain more than 80% enzyme activity under high temperature heating conditions, and the introduction of hydrophobicity significantly improves their protective effect. These findings show that such polymers have significant advantages in protecting proteins from extreme environmental impacts and are expected to be widely used in protein biopharmaceuticals and drug delivery systems.

Chapter 3

Protein protection by various type of PLL based polyampholytes

3.1 Introduction

In the second part of the study, I successfully combined polylysine (PLL) with succinic anhydride (SA) to prepare a new kind of Polyampholyte polymer, named PLL-SA.

The experimental results showed that PLL-SA showed excellent inhibition of protein aggregation. Protein aggregation is a signature pathological feature of many diseases, such as neurodegenerative diseases such as Alzheimer's and Parkinson's. Therefore, the development of materials that can effectively inhibit protein aggregation has important clinical significance. Our study found that PLL-SA showed remarkable effects in inhibiting protein aggregation, mainly due to its unique molecular structure and chemical properties.

More importantly, I further introduced hydrophobic groups based on PLL-SA. This improvement greatly improves the efficiency of the polymer in inhibiting protein aggregation. The introduction of hydrophobic groups not only enhances the interaction between the polymer and the protein, but also optimizes its dispersion and stability in the organism. The experimental data show that the inhibition effect of the improved polymer on protein aggregation is significantly better than that of the unimproved PLL-SA.

During the experiment, I observed that a polymer can significantly inhibit protein aggregation at a low concentration, which undoubtedly highlights its significant advantages in inhibiting protein aggregation. This high efficiency may result from a specific interaction between the polymer

and the protein, which effectively prevents the aggregation process of the protein at lower concentrations.

In addition, although the polymer PLL-SA (65) without the introduction of hydrophobic groups is not as effective as the modified polymer in inhibiting protein aggregation, the experimental results show that the polymer is not ideal in inhibiting protein aggregation when the SA (sulfonic acid group) content is at a very low level (Figure 3.1). This finding suggests that the regulation of SA content may be one of the key factors affecting the ability of polymers to inhibit protein aggregation. Based on this, I speculated that with the further increase of SA content, the polymer without the introduction of hydrophobic groups is also expected to show better inhibitory effect on protein aggregation.

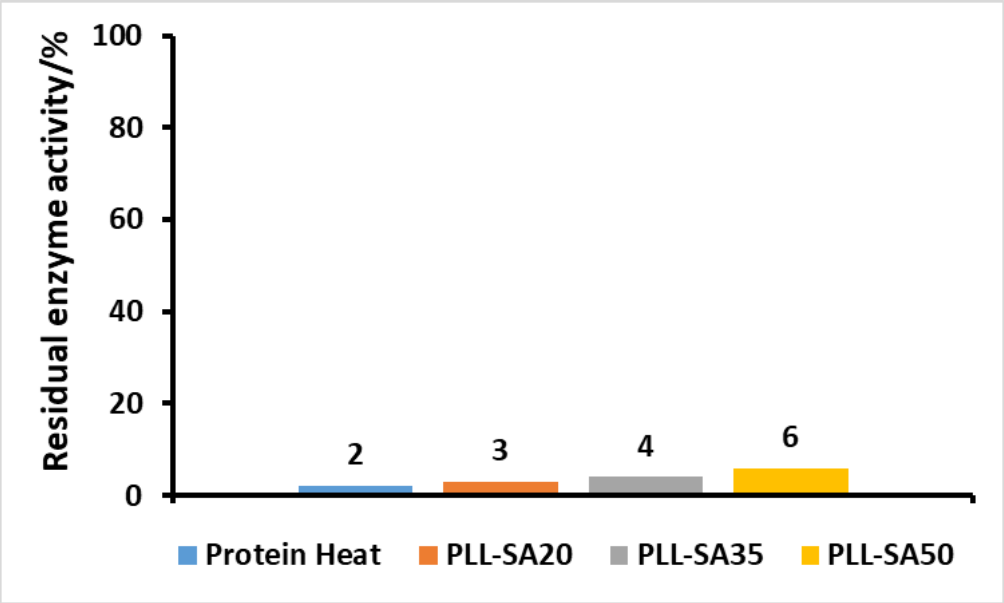


Figure 3.1 Schematic diagram of residual enzyme activity in the presence of different amounts of SA after heating.

In the following experiments, I plan to conduct in-depth research on the following four aspects to comprehensively analyze the properties and mechanisms of polymers in inhibiting protein aggregation.

First, I will systematically explore the inhibition effect of polymer on protein aggregation at different concentrations, especially at low concentrations. By precisely regulating the concentration of the polymer, I will be able to more clearly define the range of effective concentrations that inhibit protein aggregation.

Secondly, to further improve the efficiency of the polymer in inhibiting protein aggregation, I will focus on optimizing the ratio of acid anhydride to hydrophobic groups. By designing a series of polymers with different proportions and systematically evaluating their effect on inhibiting protein aggregation, I hope to find the optimal ratio of anhydride to hydrophobic groups to maximize the inhibition performance of the polymer.

Third, without introducing hydrophobic groups, I will increase the content of acid anhydride to investigate whether the polymer can still effectively inhibit protein aggregation. This research will help us understand the central role of acid anhydrides in the inhibition of protein aggregation by polymers.

Finally, to verify the effect of chemical structure on the inhibition of protein aggregation function of the polymer, I will introduce acid anhydride dimethyl glutaric anhydride (DMGA), whose structure is like SA and conduct a comparative experiment. The effect of the chemical structure on the inhibition of protein aggregation was further verified by comparison.

3.2 Materials

25% (w/w) ε-Polylysine (PLL) (molecular weight 4000) aqueous solution was purchased from JNC CORPORATION (Tokyo, Japan). SA (Succinic Anhydride) and dimethyl glutaric anhydride (DMGA) were purchased from NACALAI TESQUE, INC (Kyoto, Japan). DDSA (Dodecylsuccinic Anhydride) was purchased from TOKYO CHEMICAL INDUSTRY (Tokyo, Japan). Dulbecco PBS (-) powder was purchased from NISSUI PHARMACEUTICAL CO., LTD (Tokyo, Japan). Lysozyme from chicken egg white, ANS-NH_4, THT, Micrococcus lysodeikticus and LDH (Lactate Dehydrogenase) from rabbit muscle were purchased from Sigma-Aldrich (St. Louis, MO).

3.3 Synthesis of Polymer

3.3.1 Synthesis of PLL-SA and PLL-DDSA-SA

PLL (10 mL) was mixed with SA (1.075g, 1.365g, 1.6575g), and the mixture was heated at 70°C for 2 h. In my study, I prepared PLL-SA (55, 70, 85) by neutralizing 55%, 70%, 85% of the amino groups of PLL by SA (Figure 3.2 a)

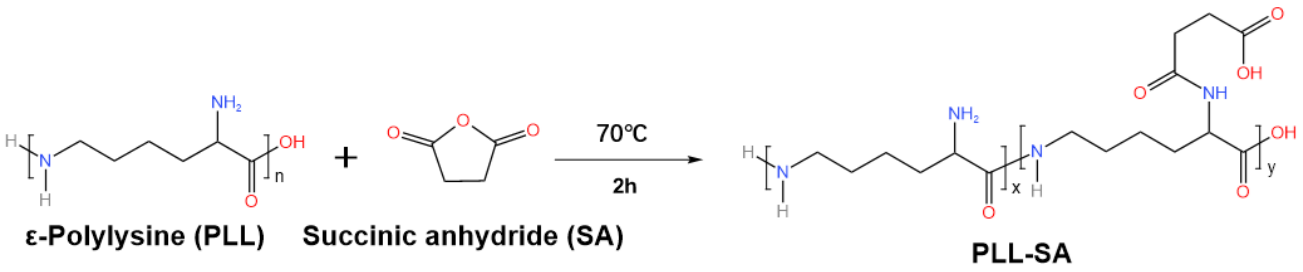


Figure 3.2 a Synthesis of ε-Polylysine with SA.

In the experiment, the hydrophobic monomer DDSA (6%, 0.314g) was first introduced into the PLL solution (10ml), and then the mixture was heated to 100 ℃ and maintained at this temperature for two hours. After that, SA (70%, 1.365g) was added to the reaction system, and the temperature gradually lowered to 70 ℃, and the reaction continued for two hours. (Figure 3.2 b)

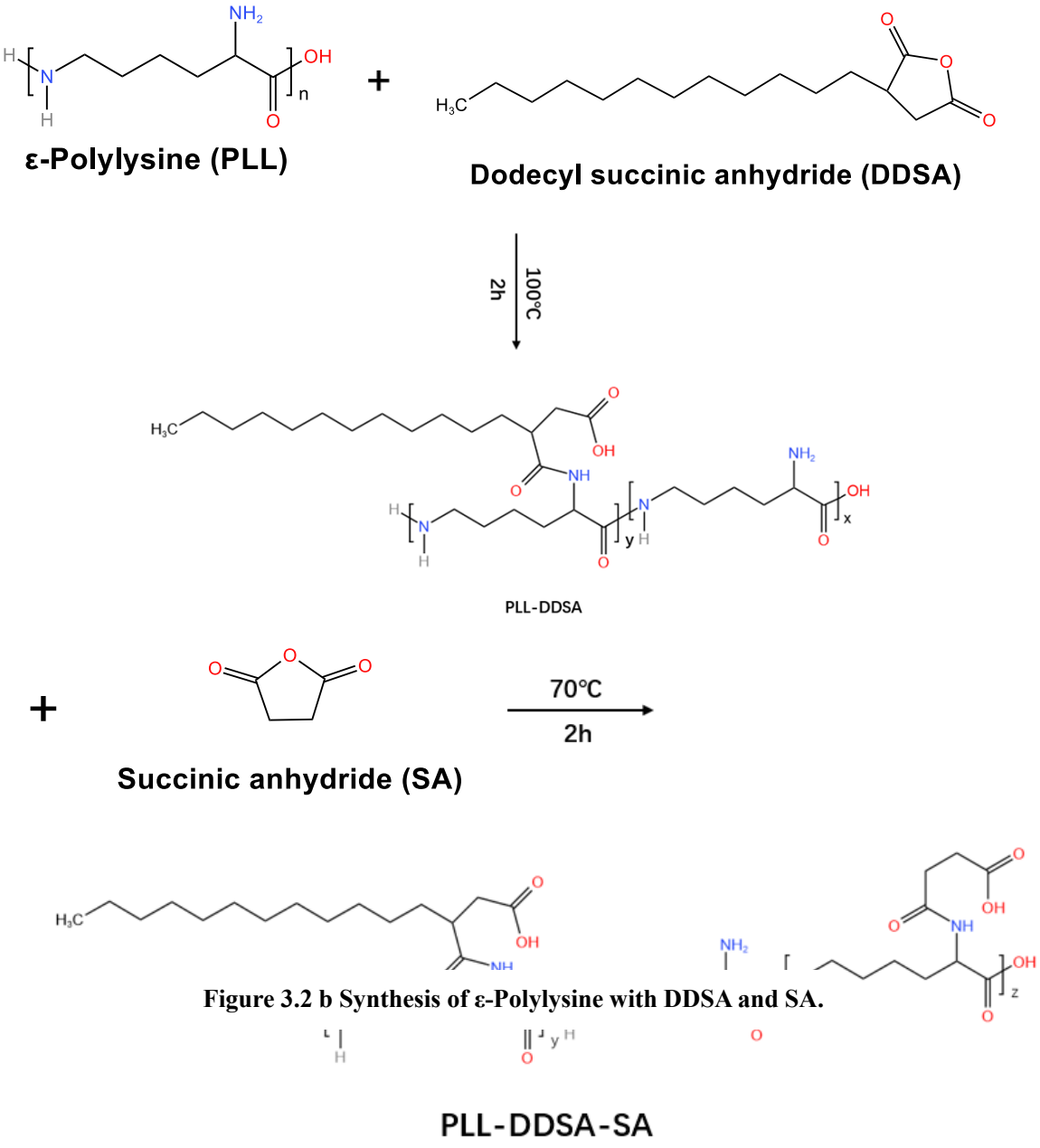


Figure 3.2 b Synthesis of ε-Polylysine with DDSA and SA.

3.3.2 Synthesis of PLL-DMGA and PLL-DDSA-DMGA

PLL (10 mL) was mixed with DMGA (1.075g, 1.365g, 1.6575g), and the mixture was heated at 70℃ for 2 h. In my study, I prepared PLL-DMGA (55, 70, 85) by neutralizing 55%, 70%, 85% of the amino groups of PLL by DMGA (Figure 3.2 c)

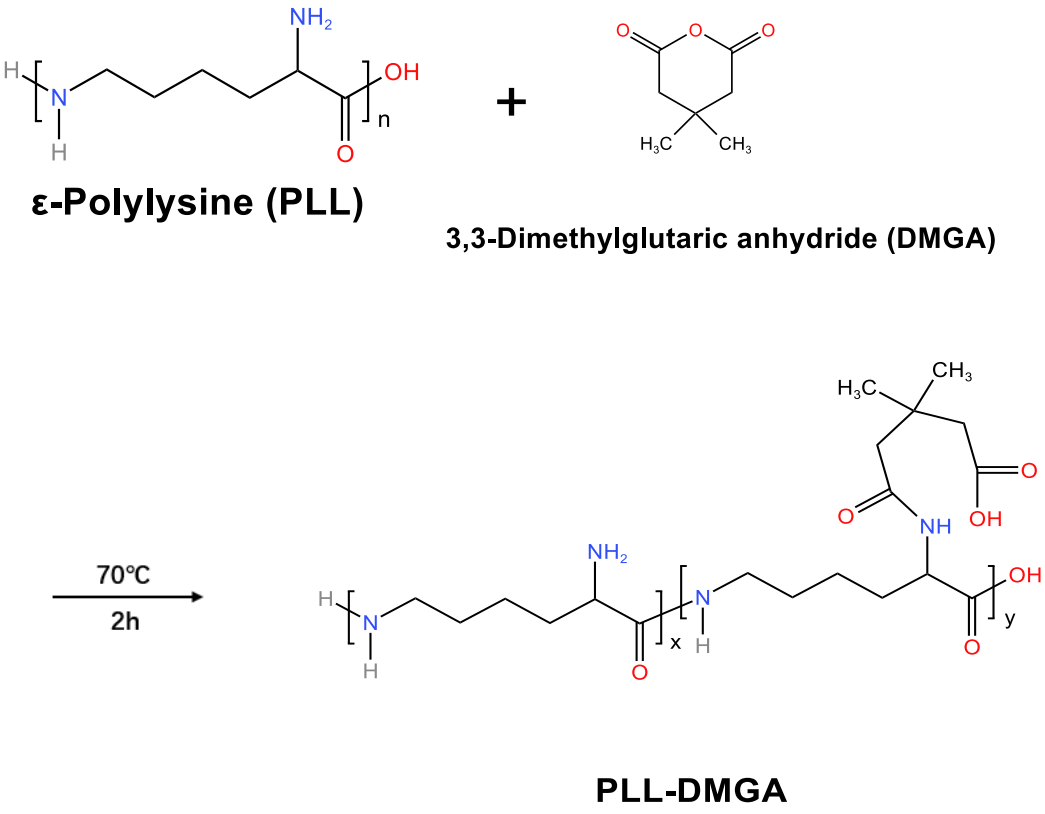


Figure 3.2 c Synthesis of ε-Polylysine with DMGA.

Next, In the experiment, the hydrophobic monomer DDSA (6%, 0.314g) was first introduced into the PLL solution (10ml), and then the mixture was heated to 100 ℃ and maintained at this temperature for two hours. After that, DMGA (70%, 1.365g) was added to the reaction system, and the temperature gradually lowered to 70 ℃, and the reaction continued for two hours. (Figure 3.2 d)

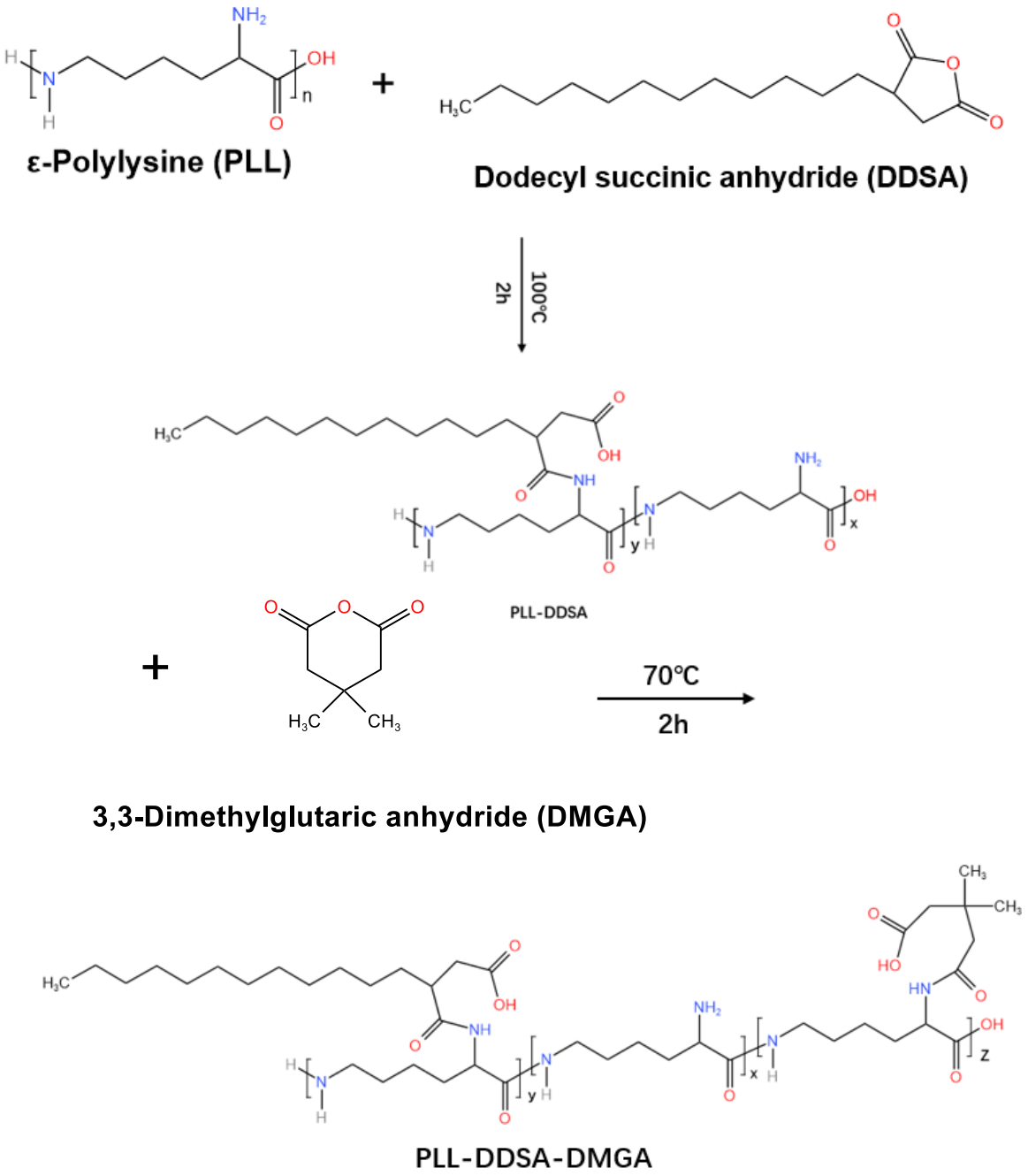


Figure 3.2 d Synthesis of ε-Polylysine with DDSA and DMGA.

3.4 Characterization of the polymers and protein

All polymers were thoroughly characterized by ^{1}H nuclear magnetic resonance spectroscopy (NMR) for an in-depth analysis of their chemical structure and composition to ensure that the resulting polymers met the expected requirements. After the sample was dried, 100mg of polymer was dissolved in 700μL D_2O, and the chemical structure of lysozyme before and after heating was detected by nuclear magnetic resonance technology. The chemical structures of all polymers were analyzed in detail using a 400 MHz Bruker AVANCE III spectrometer, and the NMR data were precisely processed and analyzed using Topspin 3.5 software.

3.5 Inhibition of protein aggregation

In the experiment, I continue to use lysozyme as a model protein to verify whether the synthesized polymer can inhibit protein aggregation. To further investigate, I systematically tested polymers at different concentrations (1mg/ml, 2mg/ml, 4mg/ml, and 8mg/ml) through gradient contrast analysis, aiming to reveal the inhibition effect of polymers on protein aggregation at different concentrations, especially at low concentrations.

Some experimental methods, such as dynamic light scattering (DLS) and residual enzyme activity, are consistent with the above content, so I will not repeat them here.

3.5.1 Absorbance measurement

In this experiment, plan to use the jasco V-770 (Tokyo, Japan) instrument to real-time monitor the absorbance changes of protein solutions and protein and polymer mixed solutions heated at 90°C for 1 hour at 600 nm wavelength. With this experiment, I will be able to gain a deeper understanding of the effect of polymers on protein stability.

3.5.2 Thioflavin T assay

Prepare Thioflavin T (ThT) solution. 4 mg of ThT was dissolved in 5 ml of PBS buffer and filtered through a 0.22-micron filter membrane to ensure the purity of the solution. Subsequently, the solution was diluted with 1 ml of ThT solution in 50 ml of PBS to prepare a working solution.

Next, the lysozyme solution and the mixture of lysozyme and polymer were heated to 90°C for 30 minutes. Then, take 100 μL of this heated solution and mix it with 2 ml of ThT working solution. The JASCO FP-6500 fluorescence spectrometer was used for observation, and the excitation wavelength was set at 440 nm and the emission wavelength at 480 nm. The significant increase in fluorescence intensity indicates that ThT successfully binds to amyloid fibers and promotes the formation of amyloid protein

3.5.3 ANS Assay

The concentration of ANS-NH_4 at 350 μM was dissolved in the PBS buffer. Subsequently, the lysozyme solution and the lysozyme-polymer mixture were heated to 90 ° C for 30 minutes. After that, 100 μL samples were extracted from each solution and mixed with 2 mL of ANS solution.

Finally, the JASCO FP-8600 spectrophotometer was used to measure the emission spectrum at the excitation wavelength of 350 nm. In the wavelength range of 405 to 700 nm, the data results of the transmitted signal are collected and recorded.

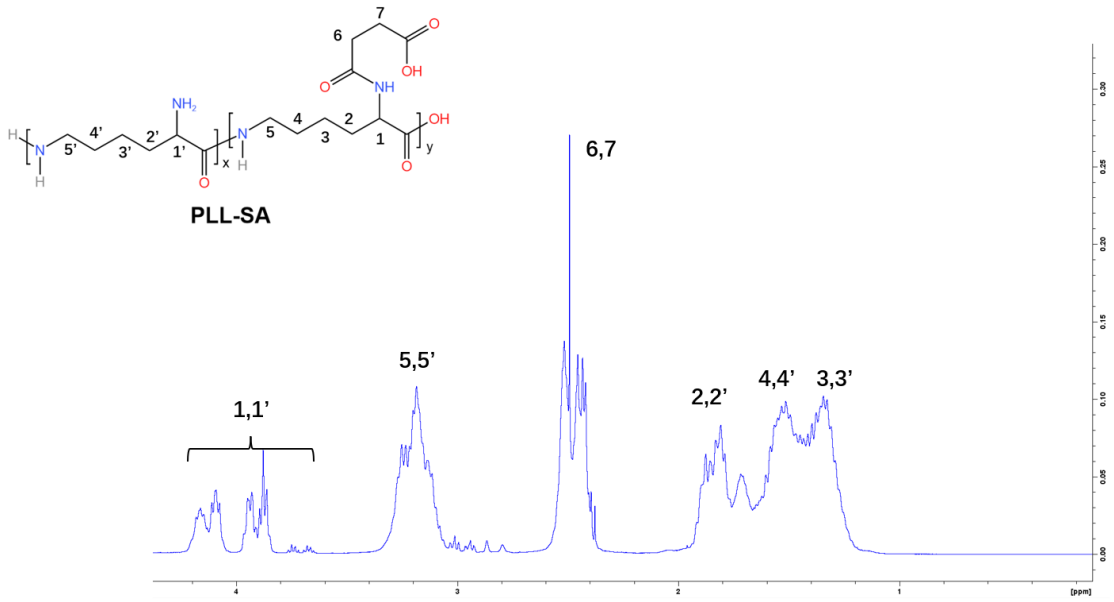
3.6 Results and discussion

3.6.1 Characterization of the PLL-SA, PLL-DDSA-SA, PLL-DMGA and PLL-DDSA-DMGA

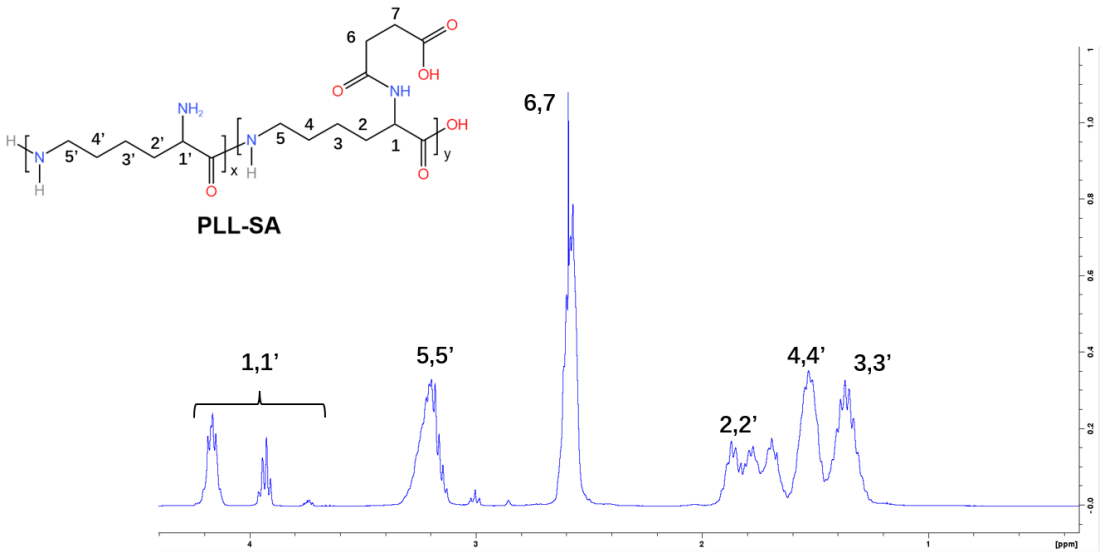
During the experiment, I successfully prepared a series of polyampholytes electrolytes by using PLL and different proportions of SA, DMGA and DDSA. These include PLL-SA (55%, 70%, 85%), PLL-DDSA (6%)-SA (70%), PLL-DMGA (55%, 70%, 85%), and PLL-DDSA (6%)-DMGA (70%). Subsequently, the chemical structure and composition of these polymers were analyzed in detail using nuclear magnetic resonance spectroscopy (NMR), the results of which are shown in Figure 3.3 (a-h).

It can be clearly observed from Figure 3.3 that the chemical shifts of hydrogen atoms in different environments for each compound have been accurately recorded. This indicates that I have successfully synthesized the expected compound. Specifically, Figure 3.3 (a), (b), and (c) show the spectra of PLL-SA (55%, 70%, 85%), respectively, while Figure 3.3 (d) shows the spectra of PLL-DDSA (6%)-SA (70%). In addition, the spectrum of PLL-DMGA (55%, 70%, 85%) are shown in Figure 3.3 (e), (f) and (g) respectively, while the spectra of PLL-DDSA (6%)-DMGA (70%) are shown in Figure 3.3 (h).

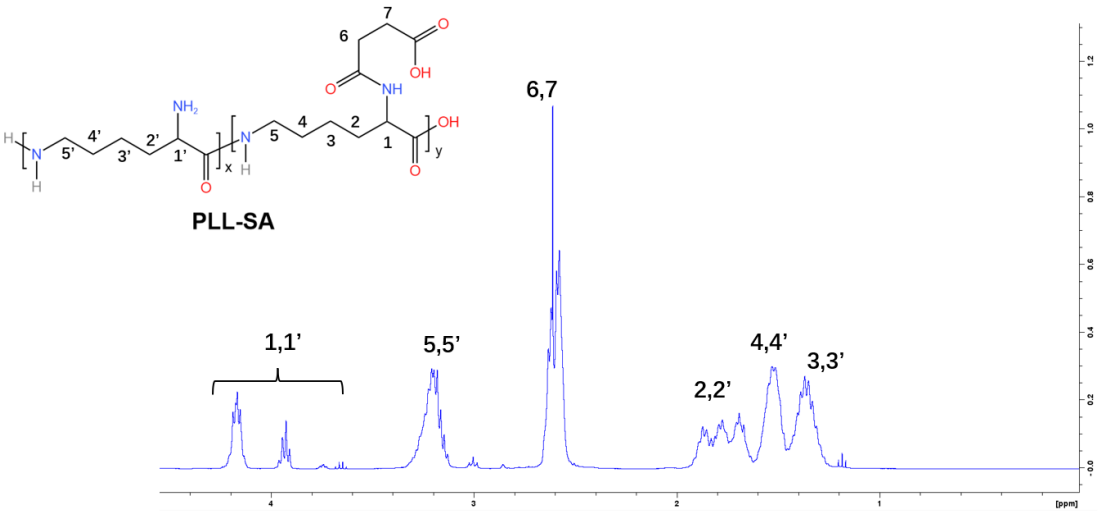
(a)



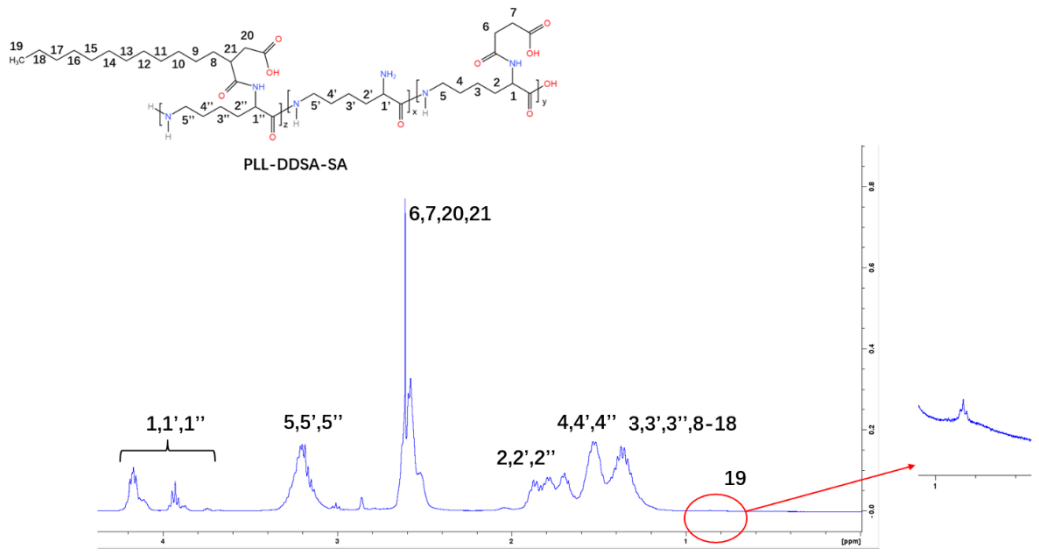
(b)



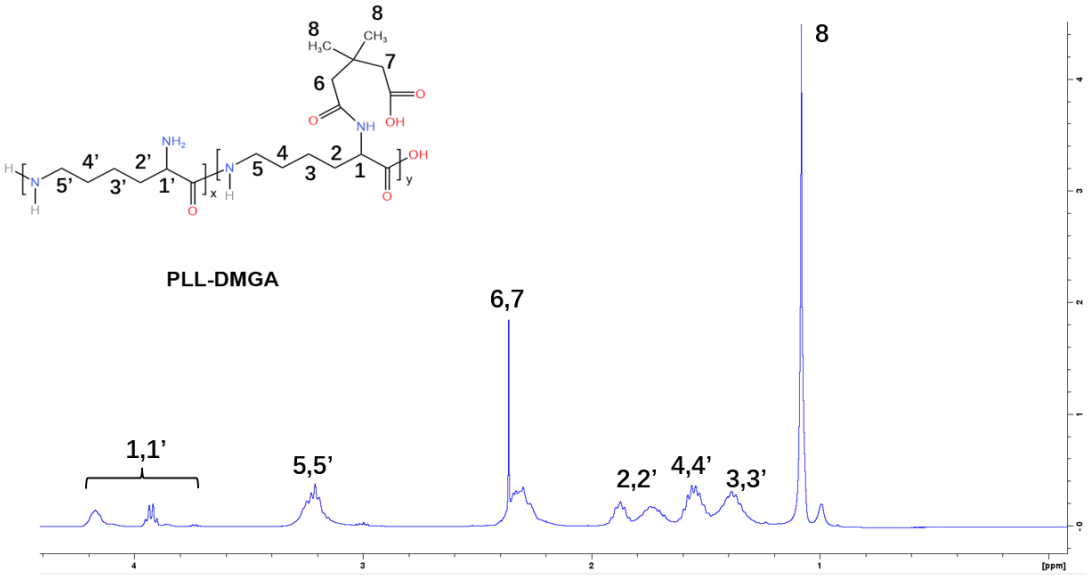
(c)


PLL-SA
6,7
1,1'
5,5'
2,2'
4,4'
3,3'
[ppm]

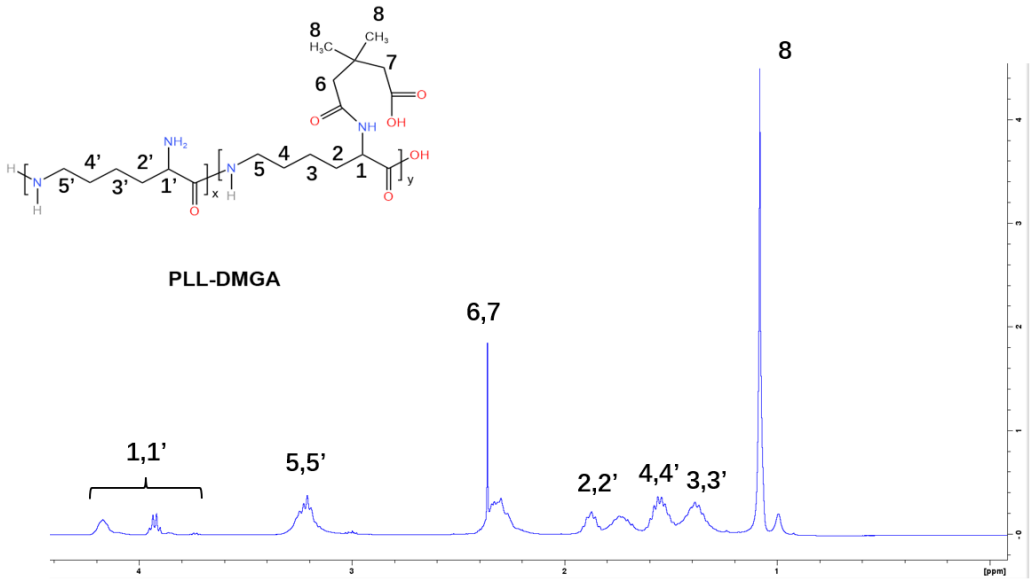
(d)


PLL-DDSA-SA
6,7,20,21
1,1',1''
5,5',5''
2,2',2''
4,4',4''
3,3',3'',8-18
19
[ppm]

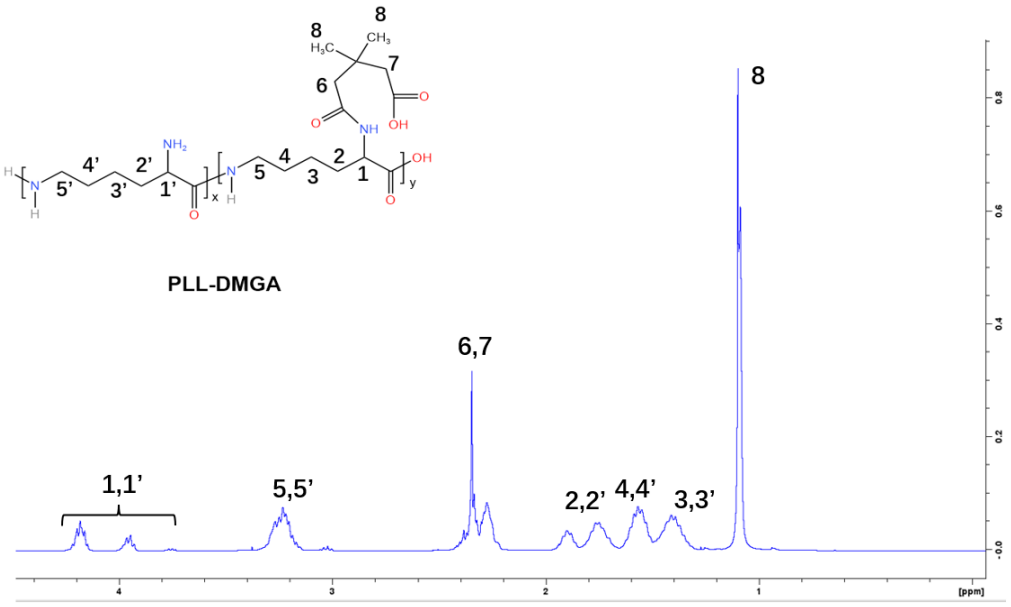
(e)



(f)



(g)



(h)

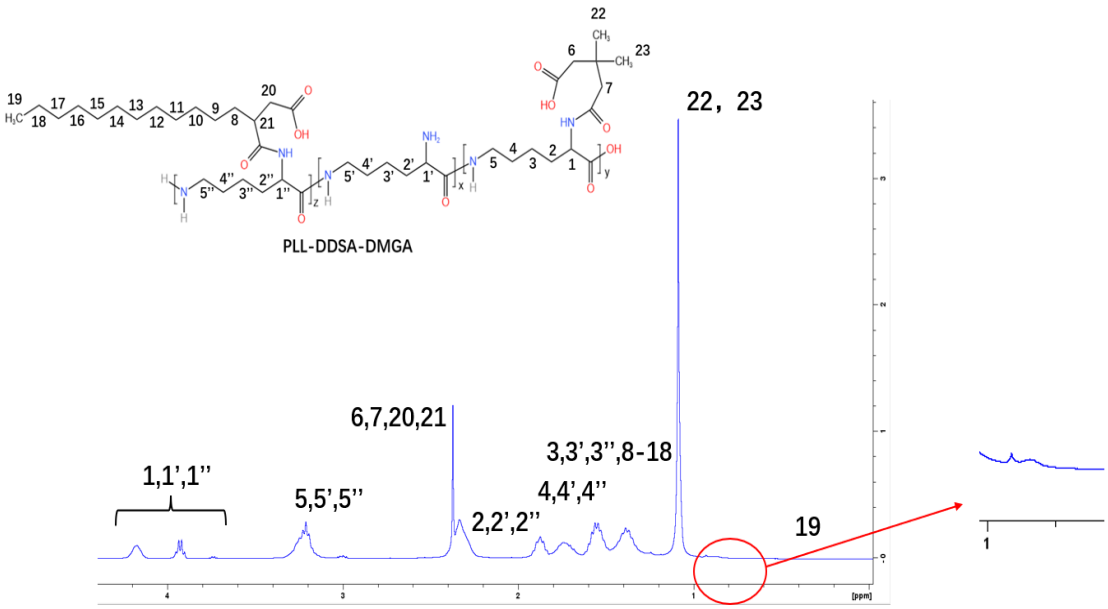
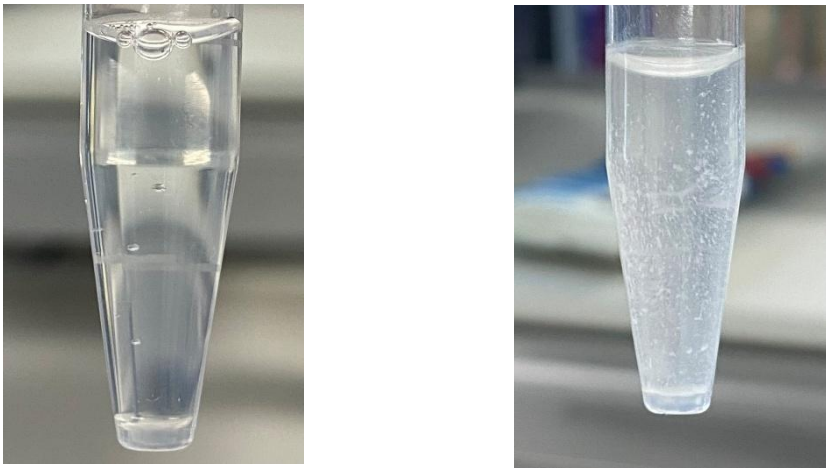


Figure 3.3 (a) (b) (c) (d) (e) (f) (g) (h) are the ^{1}H-NMR spectra of the PLL-SA (55, 70, 85) ,PLL-DDSA (6)-SA (70), PLL-DMGA(55, 70, 85) ,PLL-DDSA (6)-DMGA (70) in D2O.

3.6.2 The appearances of the lysozyme solution in comparison before and after heating.

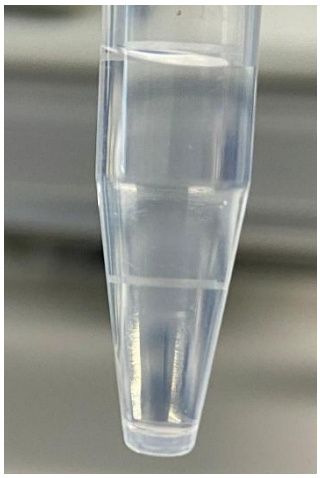
According to the experimental results in Figure. 3.4 a and b, it can be clearly observed that many white precipitates appeared in the protein solution after heating at 90℃ for 30 minutes. This phenomenon is in stark contrast to the unheated natural protein solution that remains clear and transparent in a PBS buffer (pH 7.4)



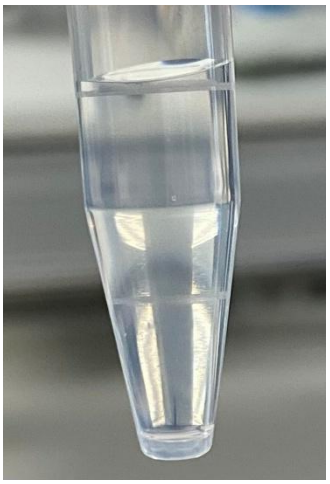
(a) **(b)**

Figure 3.4 Only lysozyme before (a) and after (b) heating 30 mins.

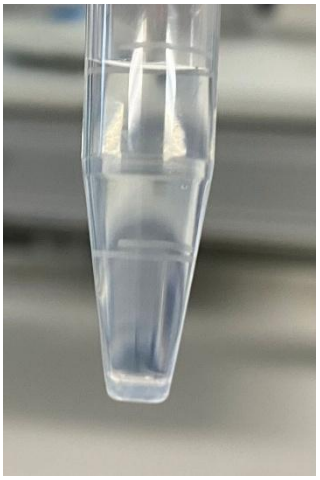
Next, I will show pictures of some of the protein solution samples, which are prepared after being mixed with different concentrations of polymer and heated for 30 minutes. Figure 3.5 (a) and (b) show samples of PLL-SA (85) at concentrations of 4 mg/ml and 8 mg/ml, respectively. Figures 3.5 (c) and (d) show samples of PLL-DMGA (85) at concentrations of 4 mg/ml and 8mg/ml, respectively. Figures 3.5 (e) and (f) show samples of PLL-DDSA (6)-SA (70) at concentrations of 4 mg/ml and 8 mg/ml. Finally, Figure 3.5 (g) and (h) show samples of PLL-DDSA (6)-DMGA (70) at concentrations of 4 mg/ml and 8 mg/ml, respectively.



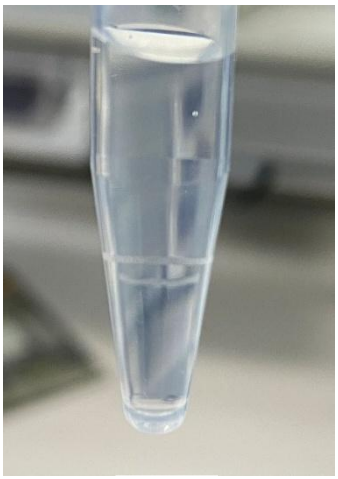
(a)



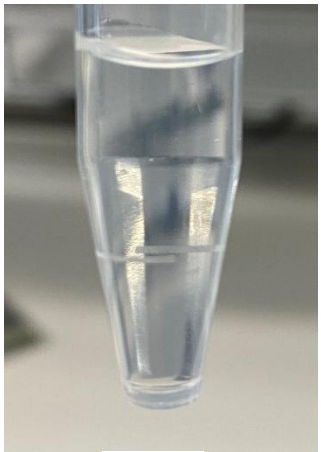
(b)



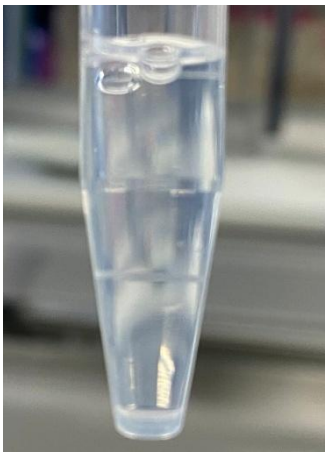
(c)



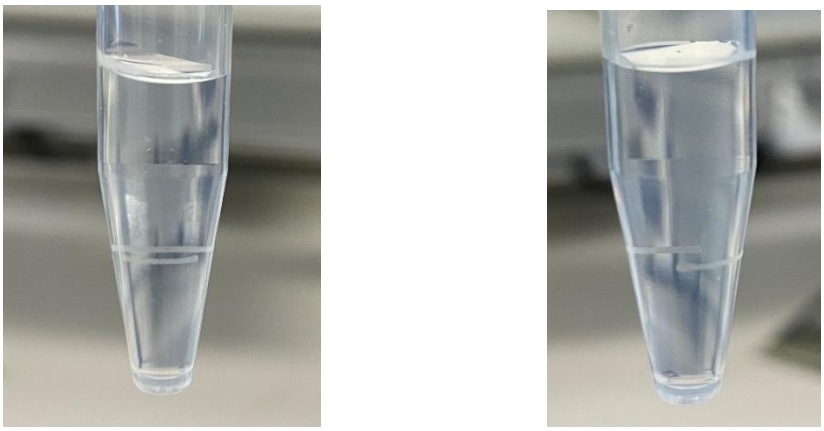
(d)



(e)



(f)



(g) **(h)**

Figure 3.5 The pictures of the PLL-SA85, PLL-DMGA85, PLL-DDSA6-SA70 and PLL-DDSA6-DMGA70 mixed with lysozyme at the different concentration after heating 30 mins.

To more accurately observe the aggregation of proteins in each solution, I used microscope technology for detailed analysis. For the experiment, I selected a BZ-X800 microscope (manufactured by Keyence Co., Ltd., Osaka, Japan). The aggregation phenomenon of different concentration solutions mixed with protein after heating was deeply observed. Figure 3.6 and Figure 3.7 show some of the experimental results.

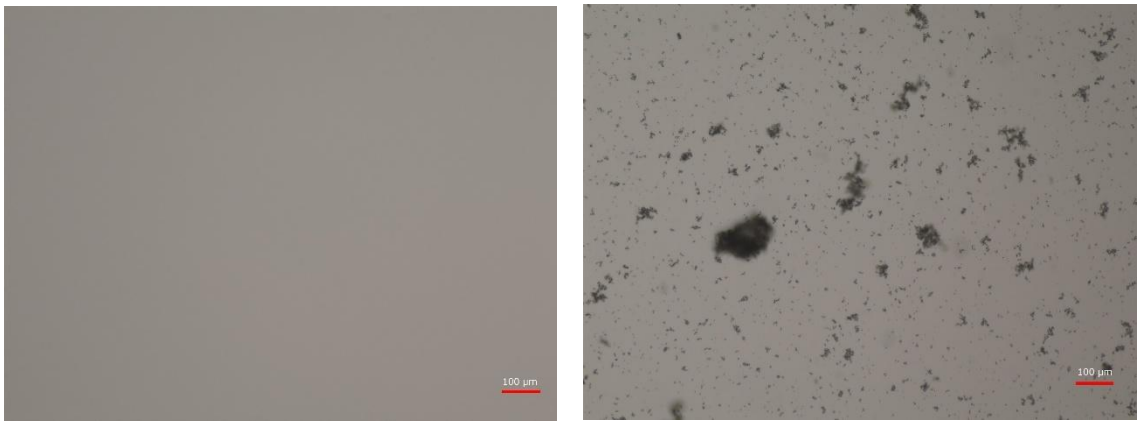


Figure 3.6 Only lysozyme before (a) and after (b) heating 30 mins. Scale bar = 100 µm.

Figure 3.7 a PLL-SA85 (4mg/ml). heating 30 mins.

Figure 3.7 b PLL-SA85 (8mg/ml). heating 30 mins.

Figure 3.7 c PLL-DMGA85 (4mg/ml). heating 30 mins.

Figure 3.7 d PLL-DMGA85 (8mg/ml). heating 30 mins.

Figure 3.7 e PLL-DDSA6-SA85 (4mg/ml). heating 30 mins.

Figure 3.7 f PLL-DDSA6-SA85 (8mg/ml). heating 30 mins.

Figure 3.7 g PLL-DDSA6-DMGA85 (4mg/ml). heating 30 mins.

Figure 3.7 h PLL-DDSA6-DMGA85 (8mg/ml). heating 30 mins.

According to the observation results of the above pictures, I can clearly draw the following conclusion: under normal conditions and in the images amplified by the microscope, the proteins after heating show a significant aggregation phenomenon. This phenomenon is often attributed to the effect of high temperatures on the interactions between protein molecules, causing irreversible changes in their structure that trigger aggregation.

However, the situation changed significantly when a specific polymer was introduced into the protein solution. Even after heat treatment, the protein can still maintain its original dispersed state. Even more remarkably, this stability is maintained even at very low concentrations (4mg/ml). This finding not only reveals the strong ability of the polymer to maintain the dispersion of the protein, but also suggests that some protective interaction between the polymer and the protein may be formed, which effectively resists the damage of the protein structure at high temperatures.

3.6.3 Dynamic Light Scattering

Next, I further examined the particle size variation of the proteins in the heated and unheated (natural) state (see Figure 3.8 a). The protein concentration used in the experiment was 0.4 mg/ml, the heating process lasted for 30 minutes, and the temperature was set at 90 ° C.

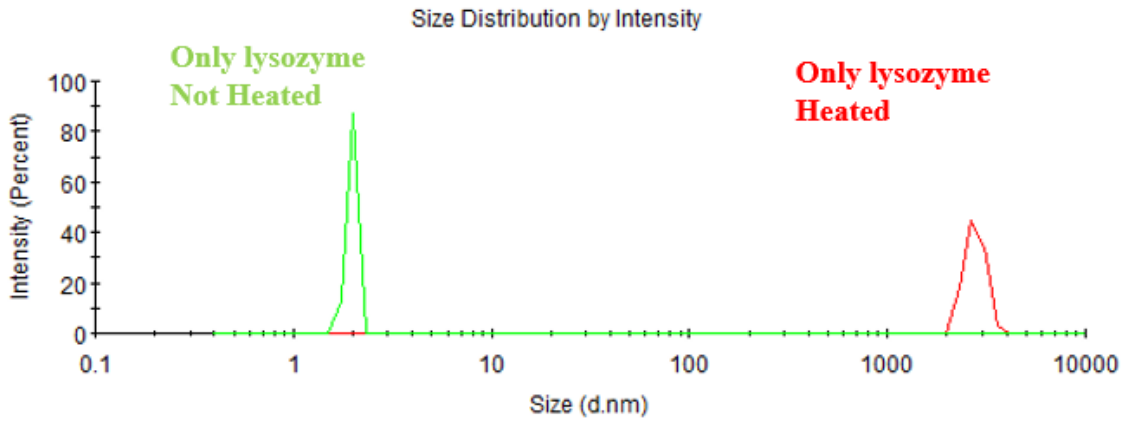


Figure 3.8 (a) The size of the protein, heated (green) and not heated (red).

It can be clearly observed from the figure 3.8 a, the protein particle size increases significantly after heating, which strongly indicates that the protein aggregation occurs during the heating process. Specifically, the heated protein particles showed a clear trend of enlargement, in sharp contrast to the unheated protein. Unheated proteins appear as very fine particles.

Next, I performed further tests on the protein-polymer mixture samples. The sample, with a protein concentration of 0.4 mg/ml and a polymer concentration of 4 mg/ml, was heated for 30 minutes. Figure 3.8b shows the particle size changes of PLL-SA (55,70,85) and PLL-DDSA (6)-SA (70) mixtures with protein after heating. Figure 3.8c shows the particle size changes of PLL-DMGA (55,70,85) and PLL-DDSA (6)-DMGA (70) mixtures with protein after heating.

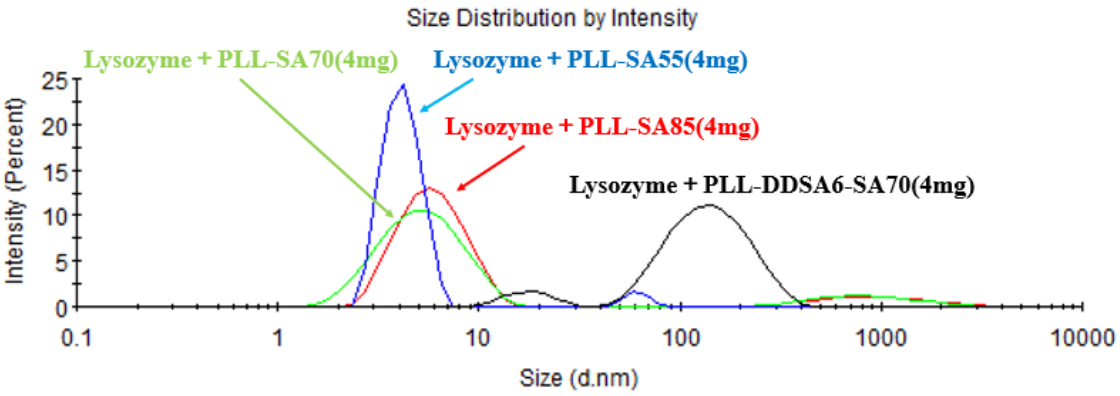


Figure 3.8 (b) The size of the mixtures, after heating 30 mins.

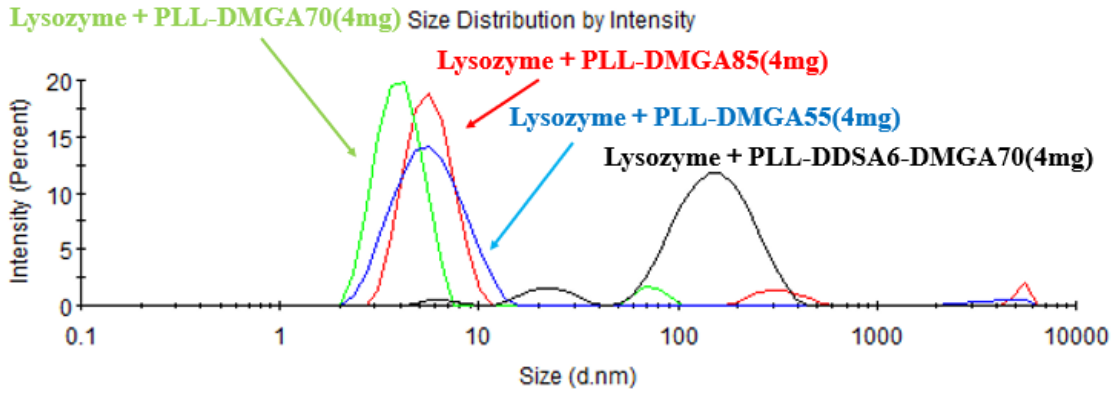


Figure 3.8 (c) The size of the mixtures, after heating 30 mins.

In studying the thermal stability of protein-mixed polymers, I observed that the particle size of these polymers can be maintained in a very small range even after high temperature treatment. This phenomenon shows that the protein-mixed polymer has excellent thermal stability and can maintain its structural integrity during heating.

Further experiments show that when hydrophobic groups are introduced and micelles may be formed, the particle size of the protein mixture polymer does not increase significantly, and the particle size is stable at about 100 nm.

To prove that the particle size increase in the sample was not caused by protein aggregation, but by the chemical structure of the polymer, I conducted the following comparative experiments, as shown in Figure 3.9 and b. The experimental results show that the particle size increase is still observed in the polymer samples even when the protein is not heated or added.

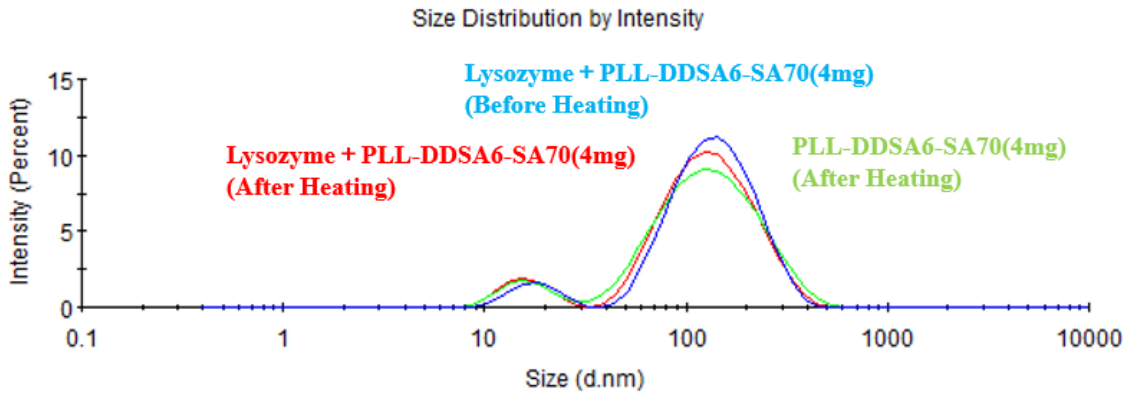


Figure 3.9 (a) The size of the mixtures.

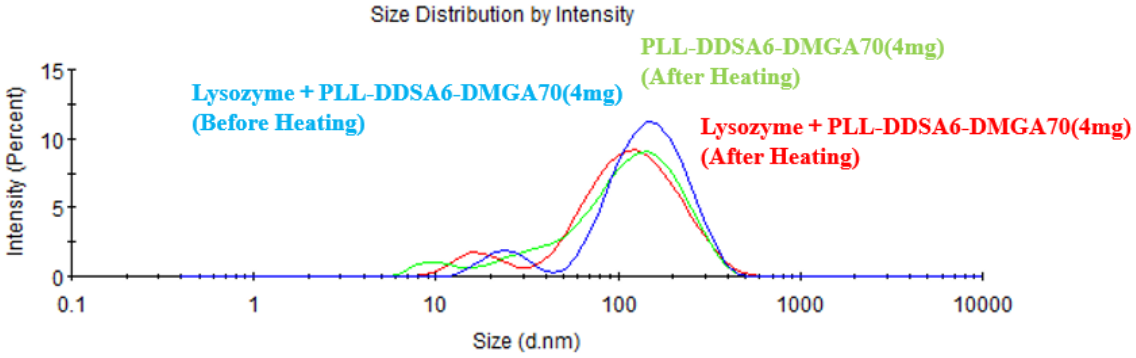


Figure 3.9 (b) The size of the mixtures.

3.6.4 Absorbance measurement of the protein solution

Proteins tend to aggregate during heating due to enhanced intermolecular interaction forces, a process that usually results in turbidity of the solution. This is because the aggregation of protein molecules causes the originally uniformly distributed protein molecules to form large aggregates in the solution, which changes the optical properties of the solution and causes the scattering of light when passing through the solution, leading to the turbidity of the appearance of the solution.

However, the situation is different when the polymer is protective of protein. The presence of polymers can effectively shield the direct interaction between protein molecules and thus inhibit protein aggregation. This protective effect allows the protein molecules to remain relatively dispersed under heating conditions, avoiding the formation of large-size aggregates. Therefore, from a macroscopic point of view, the absorbance of the solution should not change significantly.

Part of the experimental results are shown in Figure 3.10. In the experimental samples, the concentration of protein was 0.4mg/ml, and the concentration of polymer was 4mg/ml.

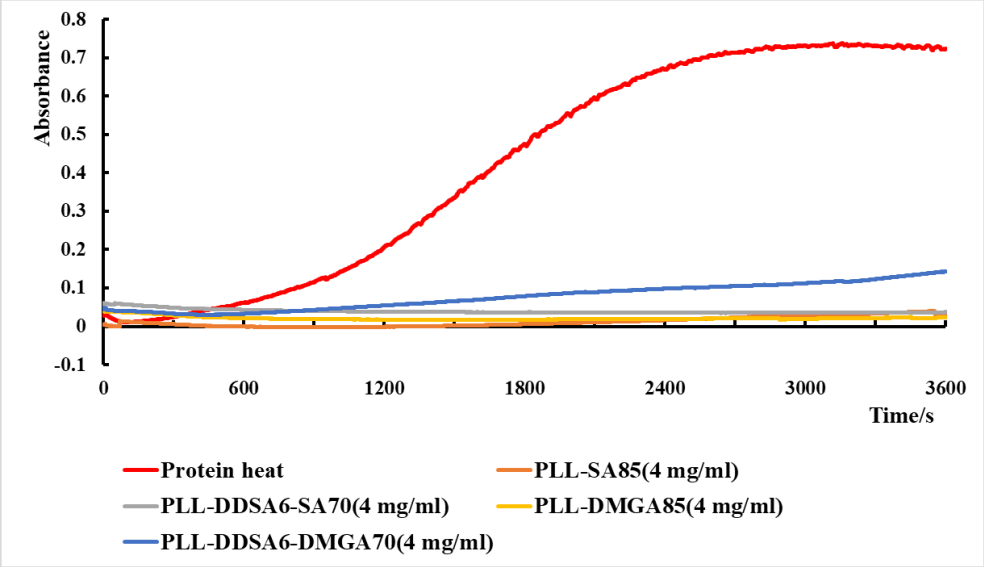


Figure 3.10 Changes in the absorbance of proteins as well as proteins mixed with polymers under continuous heating for one hour。

From the figure 3.10, it can be clearly observed that the absorbance of the protein solution gradually increased during the continuous heating for one hour, indicating that the protein began to gradually aggregate, thus causing the solution to become cloudy. However, when the polymer was added to the protein solution, no similar phenomenon was observed under the same heating condition. This result indicates that the presence of polymers can effectively maintain the stability of the protein structure and thus avoid its aggregation.

3.6.5 Residual enzymatic activity

Next, the residual enzyme activities were determined to explore whether the lysozyme in the samples could still maintain its biological activity after heat treatment.

First, I examined the enzyme activity in the unheated state to see if the intervention of the polymer influenced the lysozyme itself. The result is shown in figure 3.11 a, b.

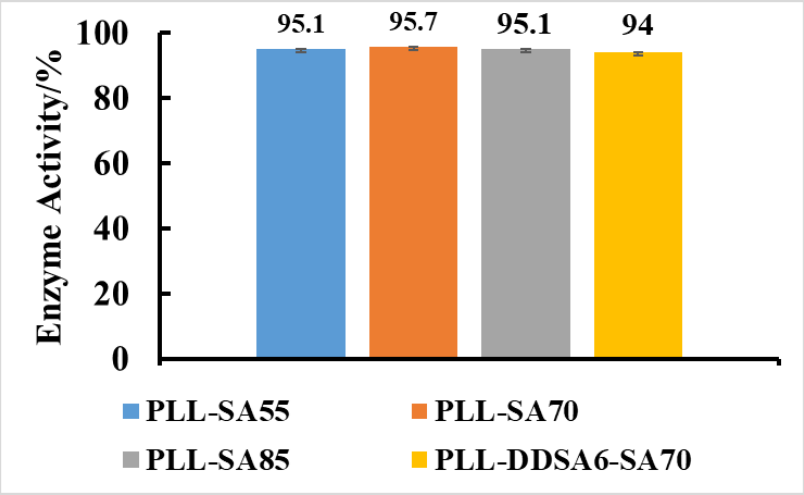


Figure 3.11 a The unheated lysozyme activity changes in the presence of the polymer.

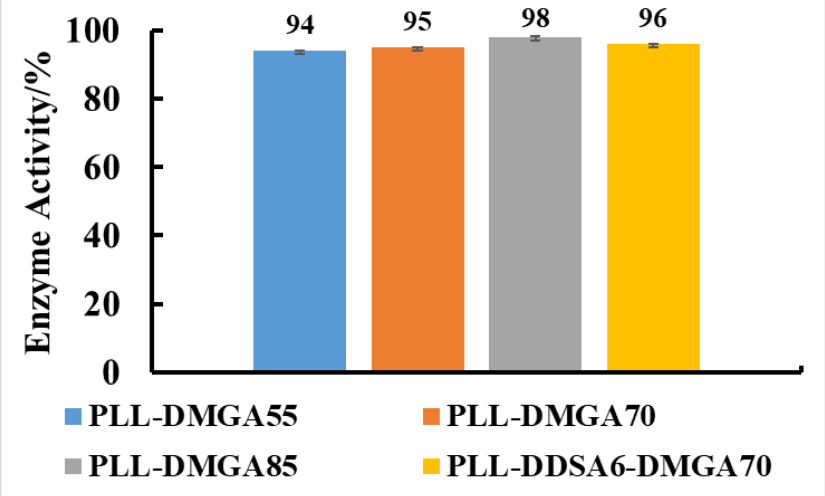


Figure 3.11 b The unheated lysozyme activity changes in the presence of the polymer.

It can be seen from the figure that the presence of polymer hardly has a great impact on the activity of lysozyme

Figure 3.12 shows the change of residual enzyme activity of PLL-SA (55,70,85) three polymer proteins after heating for 30 minutes. Where, (a), (b) and (c) correspond to the experimental results of mixing proteins at different concentrations of these three different polymers, respectively.

a

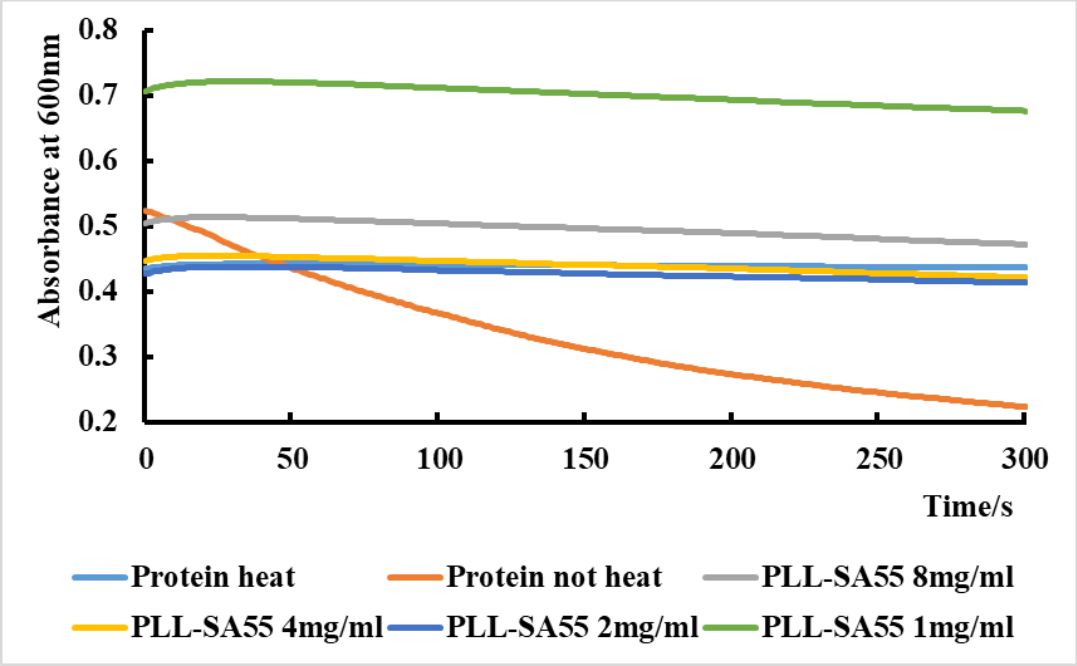


Figure 3.12 a PLL-sa55 at different concentrations.

b

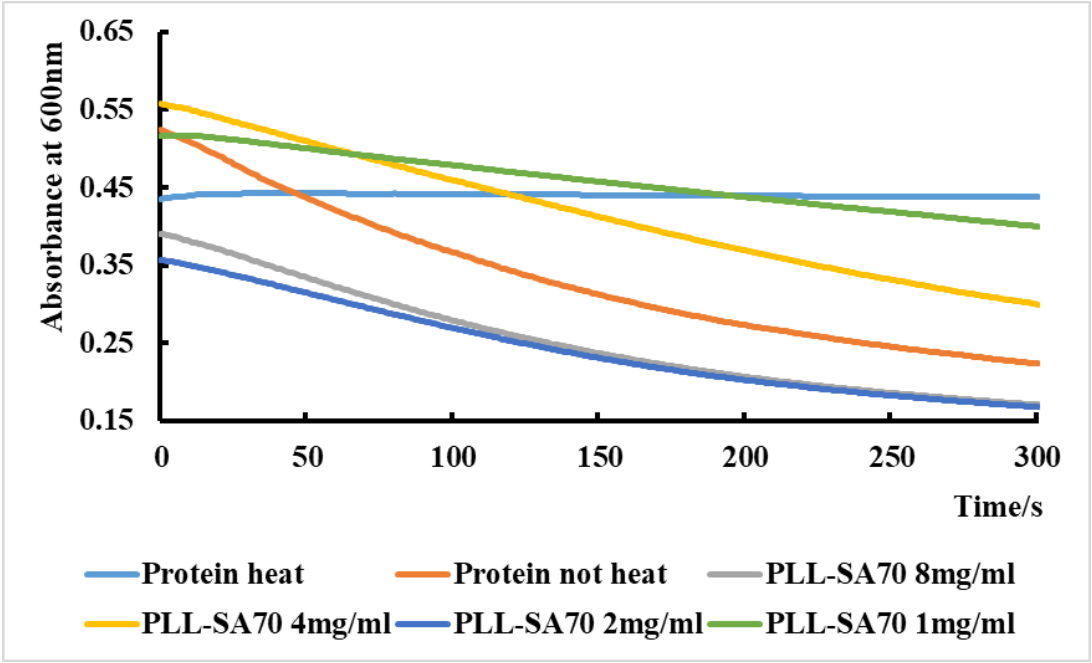


Figure 3.12 b PLL-sa70 at different concentrations.

c

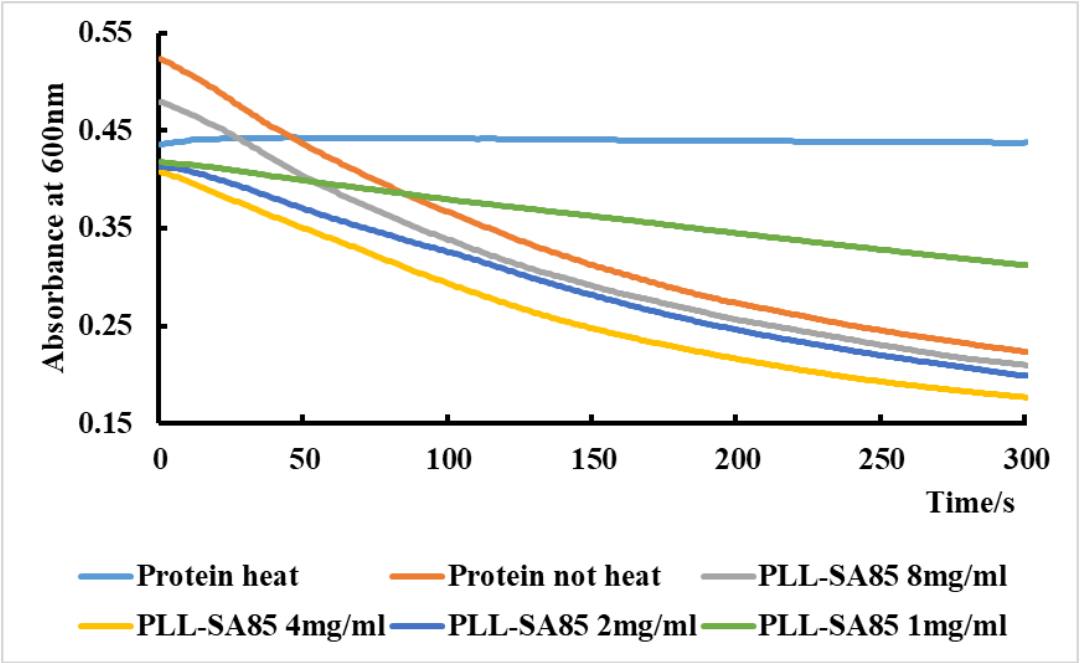


Figure 3.12 c PLL-sa85 at different concentrations.

Figure 3.13 shows the change of residual enzyme activity of PLL-DMGA (55,70,85) three polymer proteins after heating for 30 minutes. Where, (a), (b) and (c) correspond to the experimental results of mixing proteins at different concentrations of these three different polymers, respectively.

a

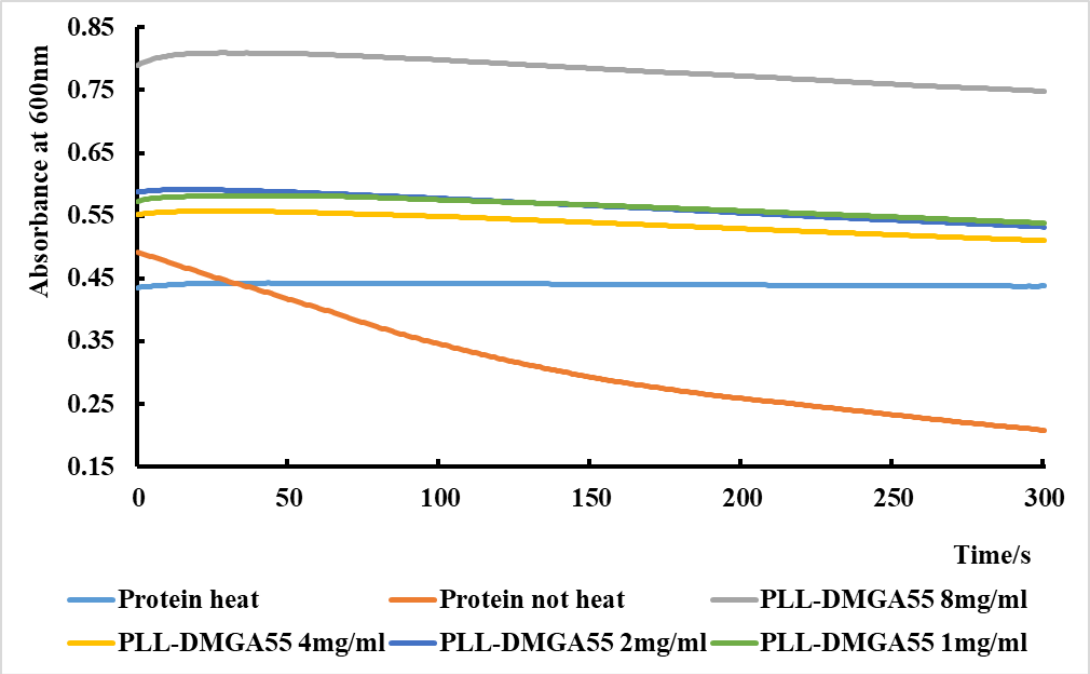


Figure 3.13 a PLL-DMGA55 at different concentrations.

b

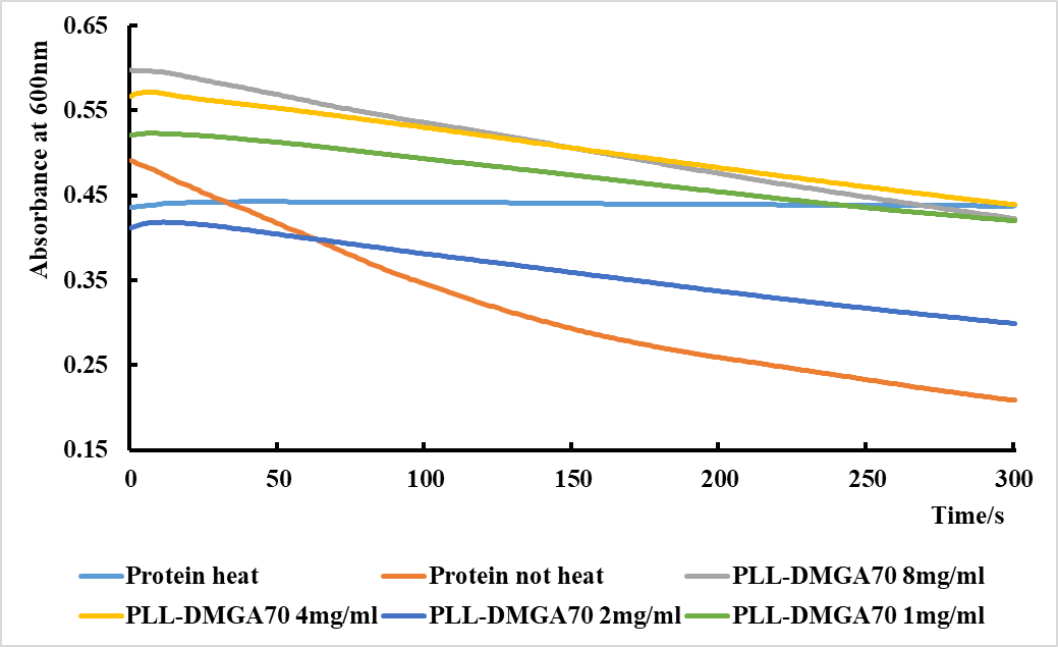


Figure 3.13 b PLL-DMGA70 at different concentrations.

c

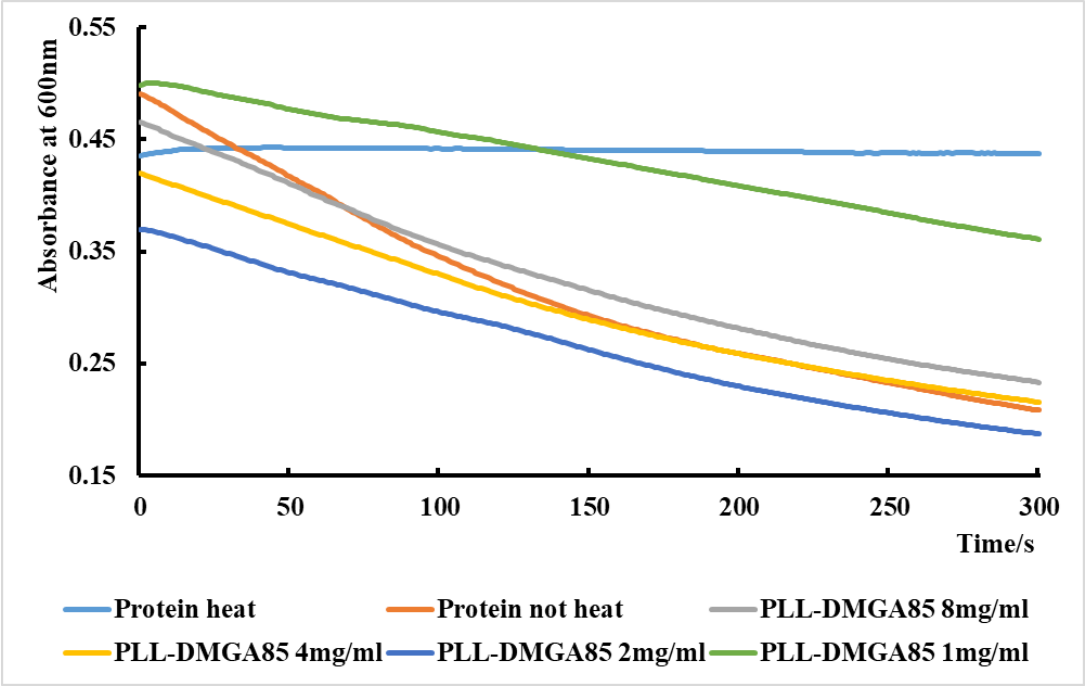


Figure 3.13 c PLL-DMGA85 at different concentrations.

Figure 3.14 shows the change of residual enzyme activity of PLL-DDSA (6)-SA, DMGA (70) two polymer proteins after heating for 30 minutes. (a) and (b)correspond to the experimental results of mixing proteins at different concentrations of these two different polymers, respectively.

a

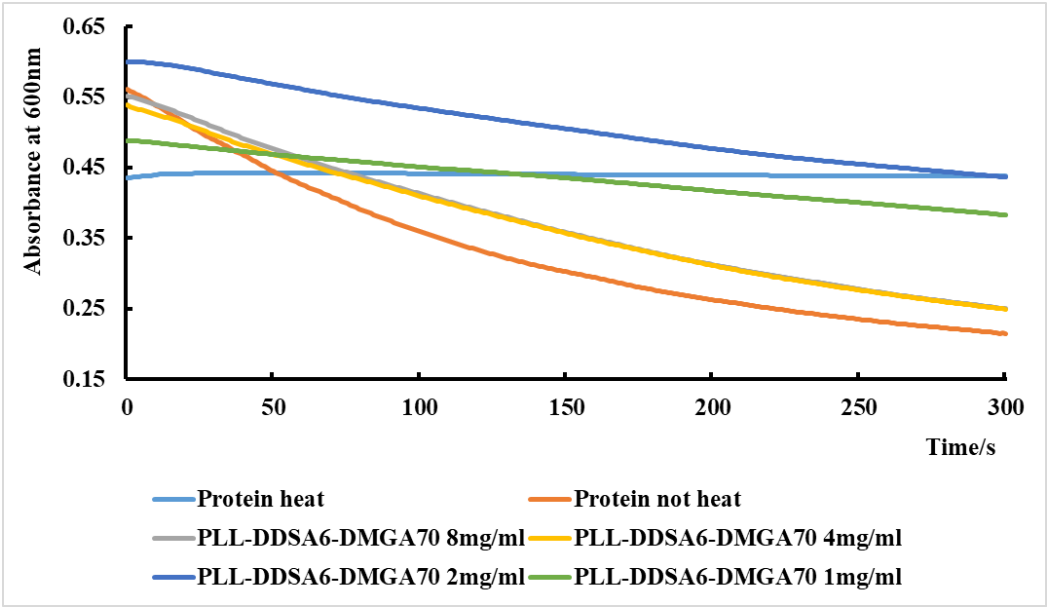


Figure 3.14 a PLL-DDSA6-DMGA70 at different concentrations.

b

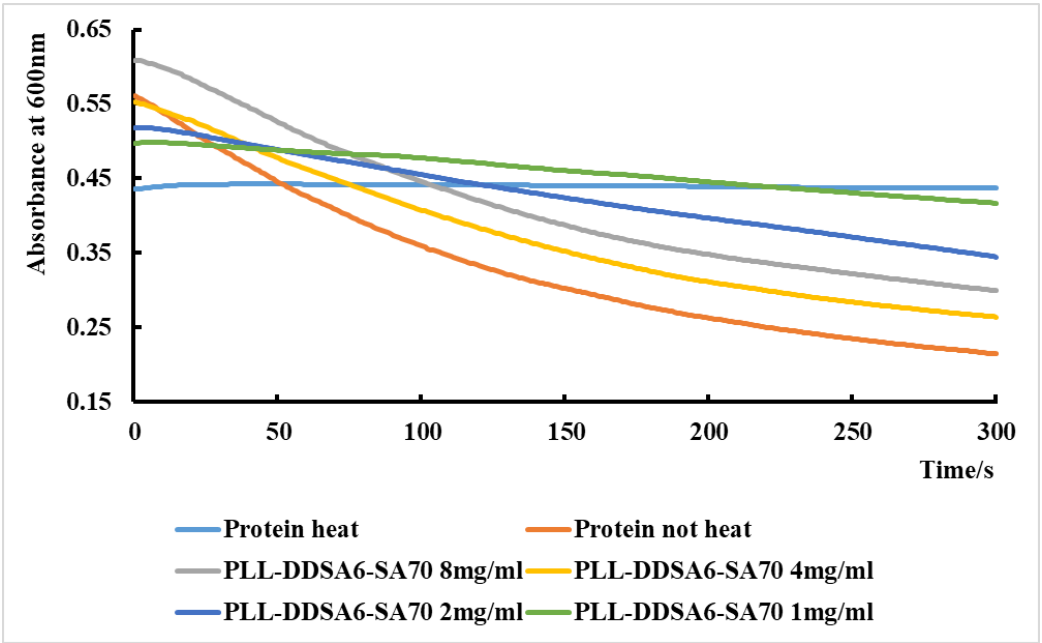
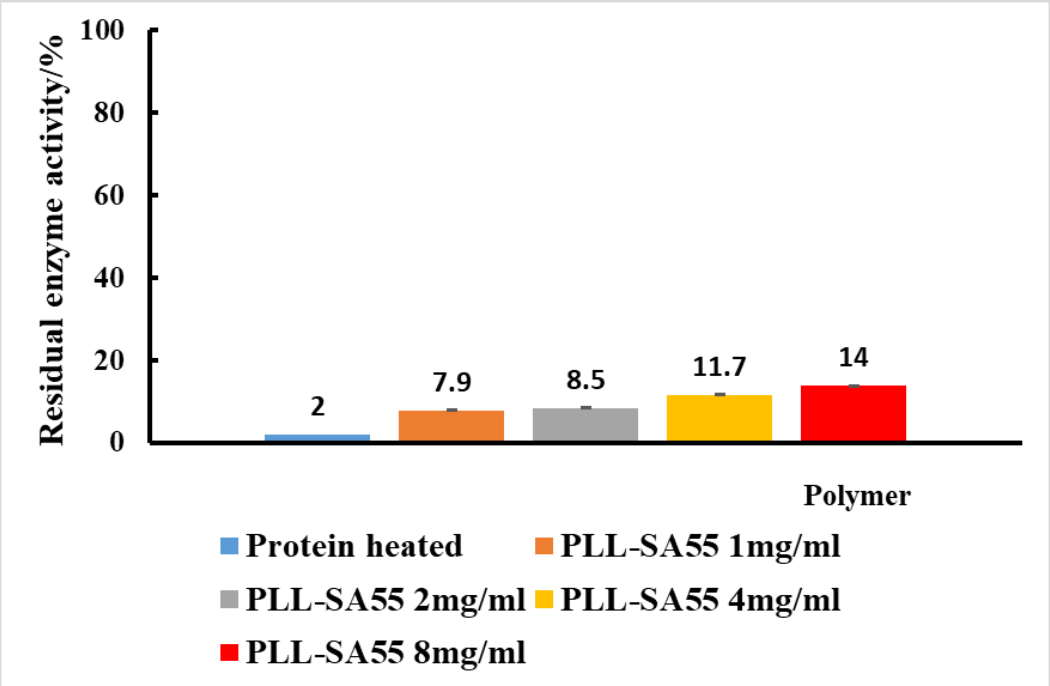


Figure 3.14 b PLL-DDSA6-SA70 at different concentrations.

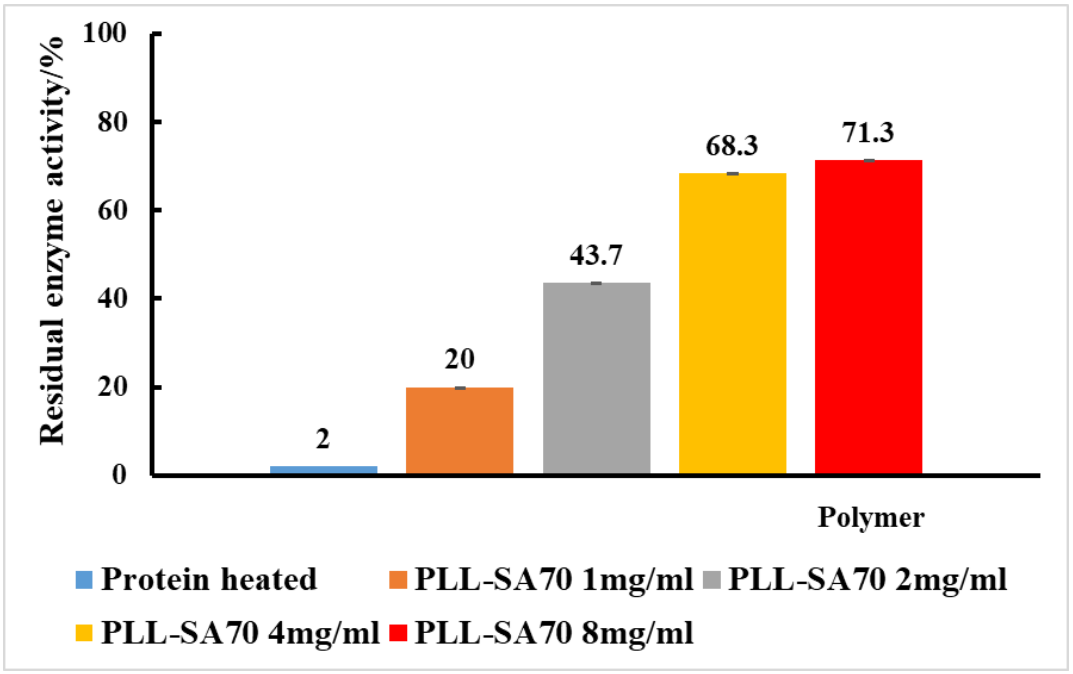
It can be seen from the figure that after adding the polymer and being heated, the absorbance of the solution still shows a downward trend as the unheated protein solution, which indicates that the treated protein can still maintain a certain activity. It is worth noting that this downward trend is particularly significant when there is a high proportion of anhydride and hydrophobic groups in the solution, and even at lower concentrations, the protein is still able to maintain a certain activity.

To compare the experimental results more conveniently, I made the experimental results into a bar chart to compare the experimental results of different polymers at different concentrations. All the results are presented in Figure 3.15

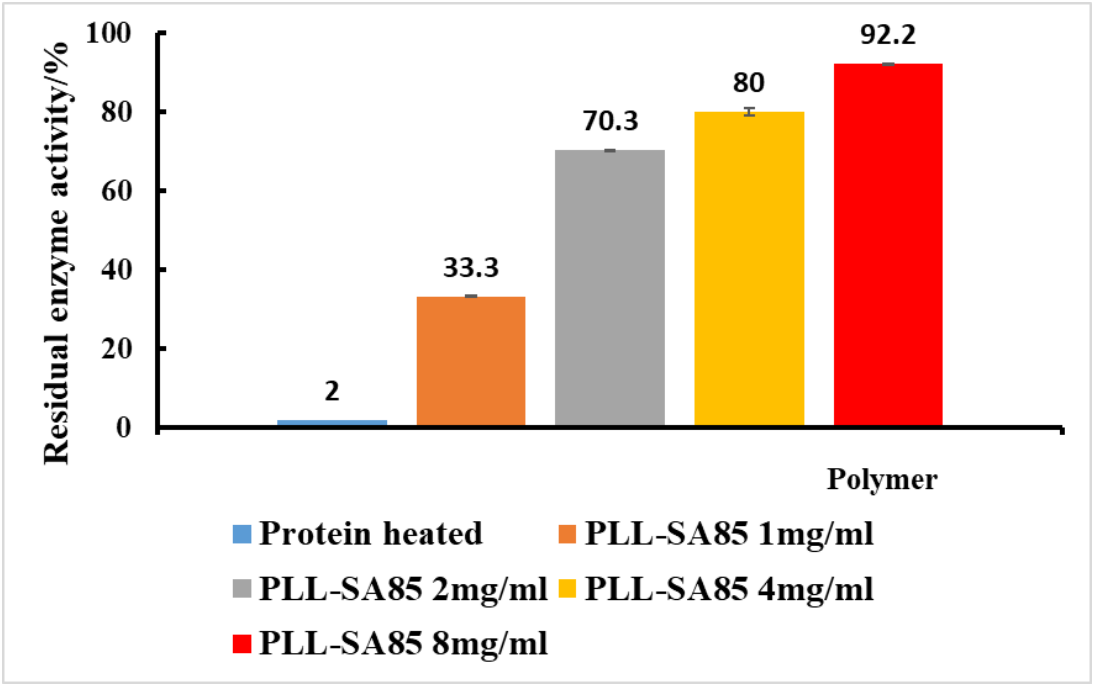
a



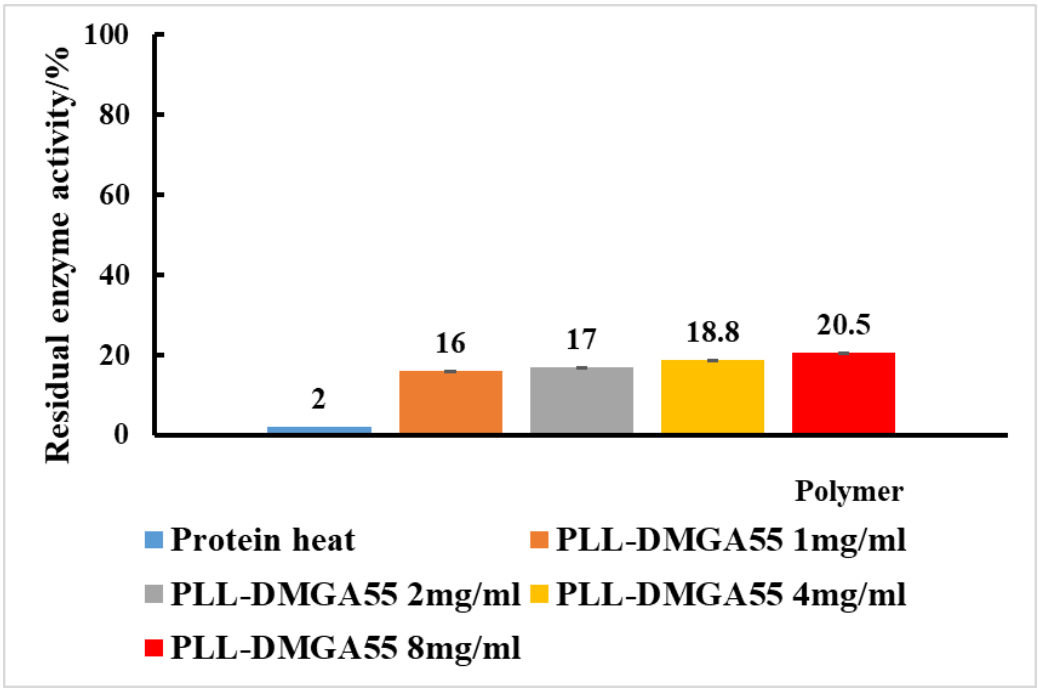
b


Residual enzyme activity/%
100
80
60
40
20
0
2
20
43.7
68.3
71.3
Polymer
Protein heated
PLL-SA70 1mg/ml
PLL-SA70 2mg/ml
PLL-SA70 4mg/ml
PLL-SA70 8mg/ml

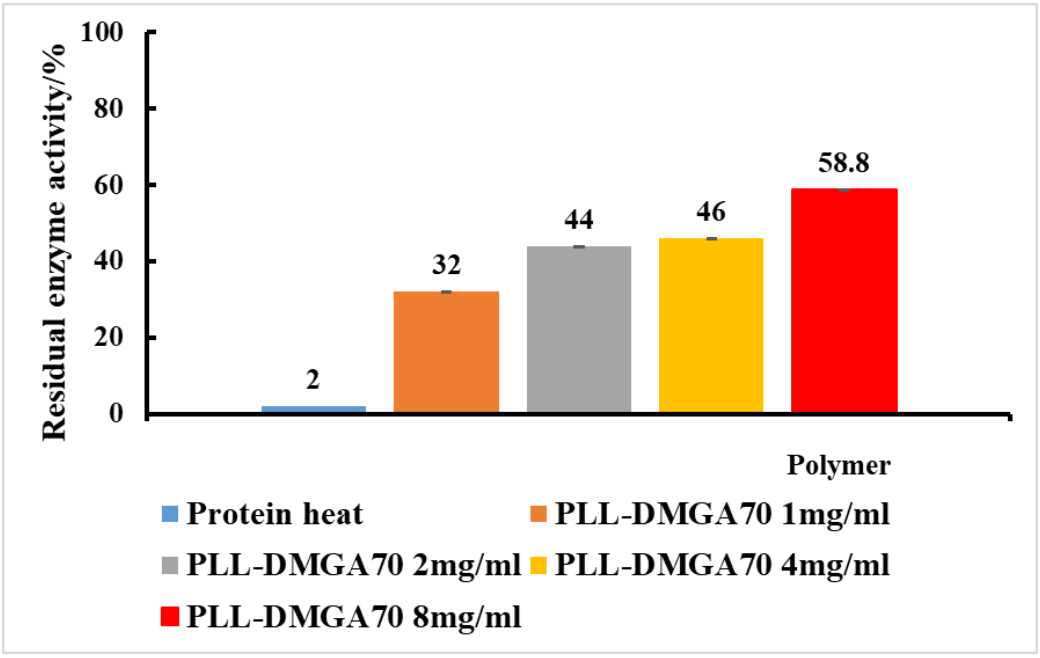
c


Residual enzyme activity/%
100
80
60
40
20
0
2
33.3
70.3
80
92.2
Polymer
Protein heated
PLL-SA85 1mg/ml
PLL-SA85 2mg/ml
PLL-SA85 4mg/ml
PLL-SA85 8mg/ml

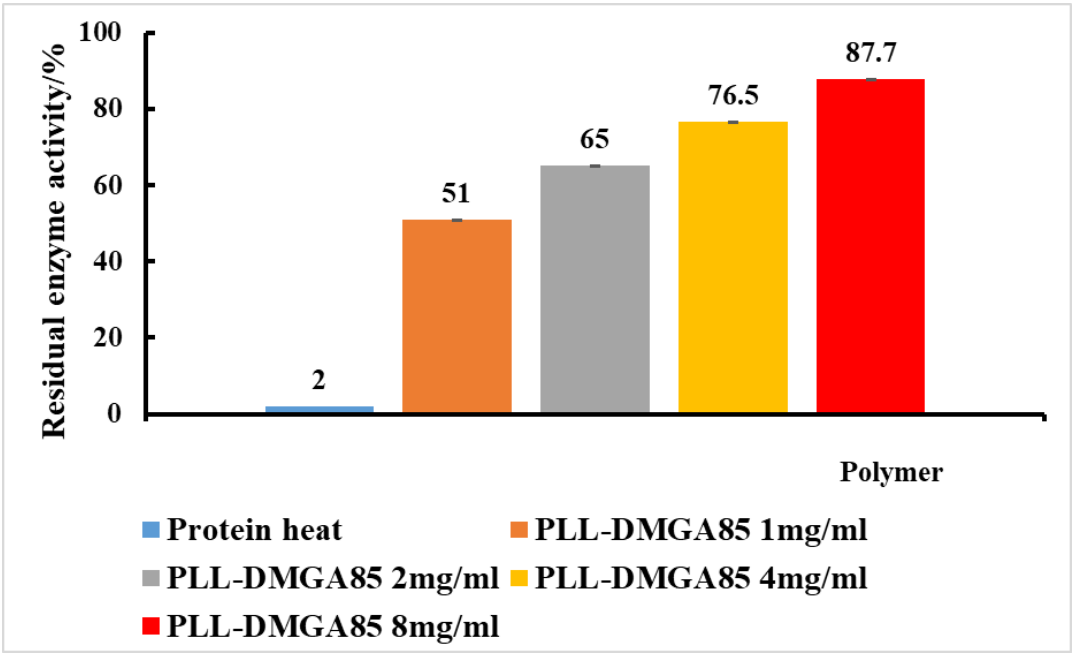
d


Residual enzyme activity/%
100
80
60
40
20
0
2
16
17
18.8
20.5
Polymer
Protein heat
PLL-DMGA55 1mg/ml
PLL-DMGA55 2mg/ml
PLL-DMGA55 4mg/ml
PLL-DMGA55 8mg/ml

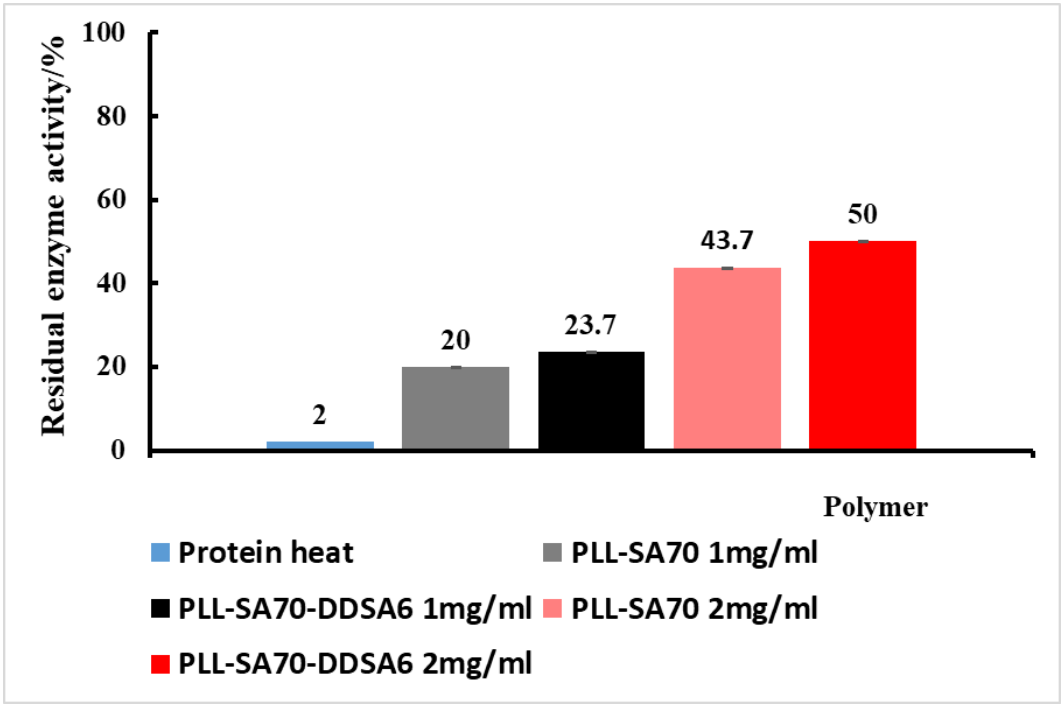
e


Residual enzyme activity/%
100
80
60
40
20
0
2
32
44
46
58.8
Polymer
Protein heat
PLL-DMGA70 1mg/ml
PLL-DMGA70 2mg/ml
PLL-DMGA70 4mg/ml
PLL-DMGA70 8mg/ml

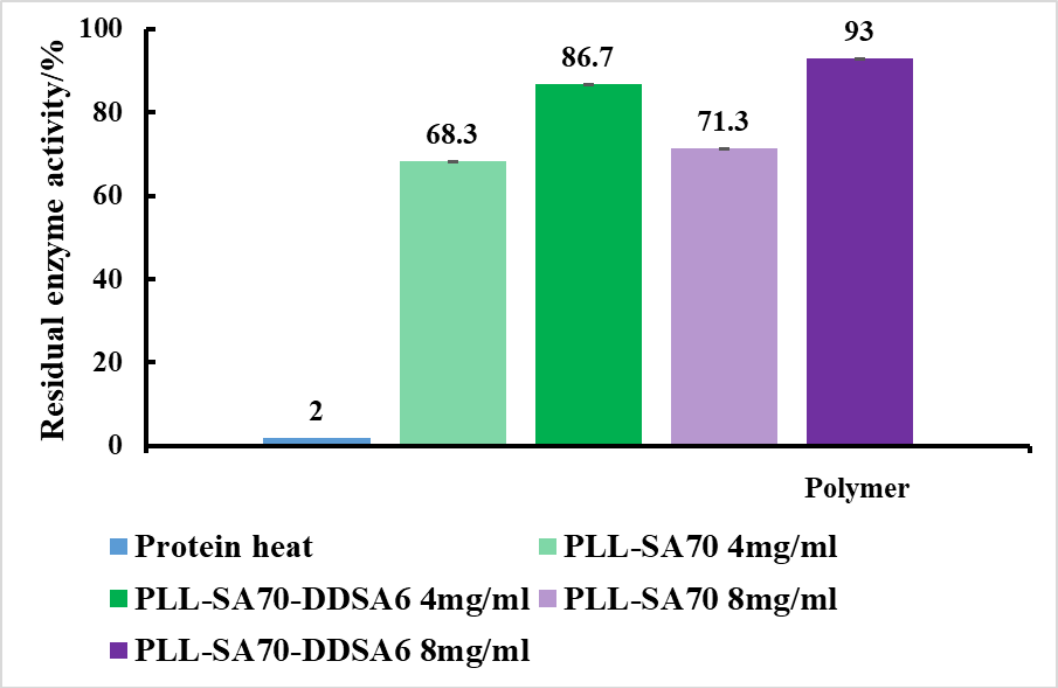
f


Residual enzyme activity/%
100
80
60
40
20
0
2
51
65
76.5
87.7
Polymer
Protein heat
PLL-DMGA85 1mg/ml
PLL-DMGA85 2mg/ml
PLL-DMGA85 4mg/ml
PLL-DMGA85 8mg/ml

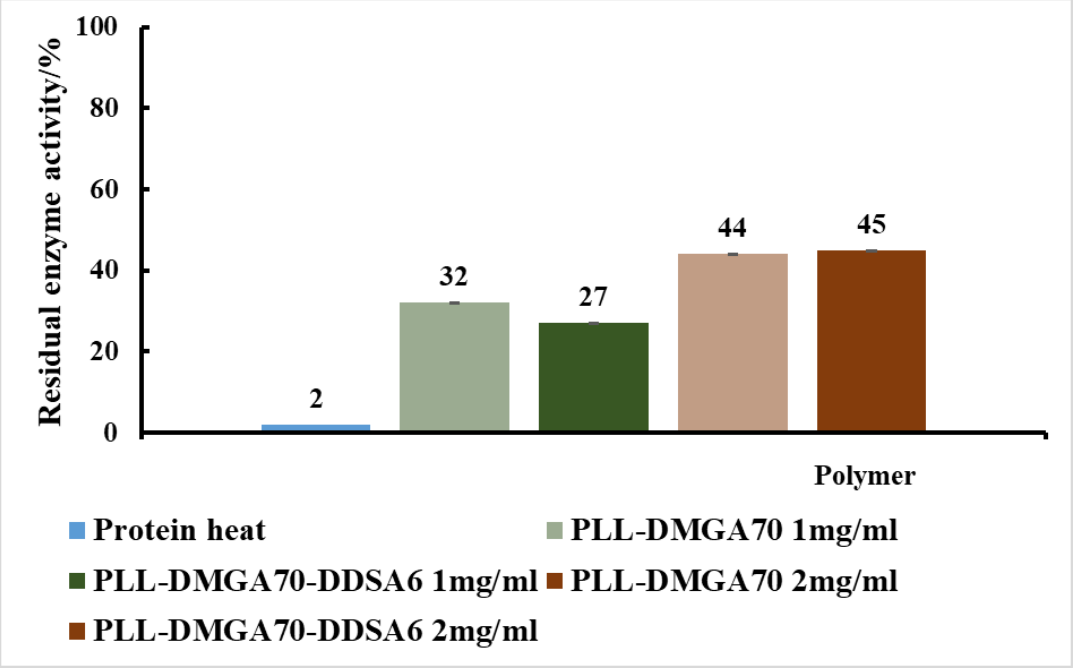
g


Residual enzyme activity/%
100
80
60
40
20
0
2
20
23.7
43.7
50
Polymer
Protein heat
PLL-SA70 1mg/ml
PLL-SA70-DDSA6 1mg/ml
PLL-SA70 2mg/ml
PLL-SA70-DDSA6 2mg/ml

h


Residual enzyme activity/%
100
80
60
40
20
0
2
68.3
86.7
71.3
93
Polymer
Protein heat
PLL-SA70 4mg/ml
PLL-SA70-DDSA6 4mg/ml
PLL-SA70 8mg/ml
PLL-SA70-DDSA6 8mg/ml

i


Residual enzyme activity/%
100
80
60
40
20
0
2
32
27
44
45
Polymer
Protein heat
PLL-DMGA70 1mg/ml
PLL-DMGA70-DDSA6 1mg/ml
PLL-DMGA70 2mg/ml
PLL-DMGA70-DDSA6 2mg/ml

j

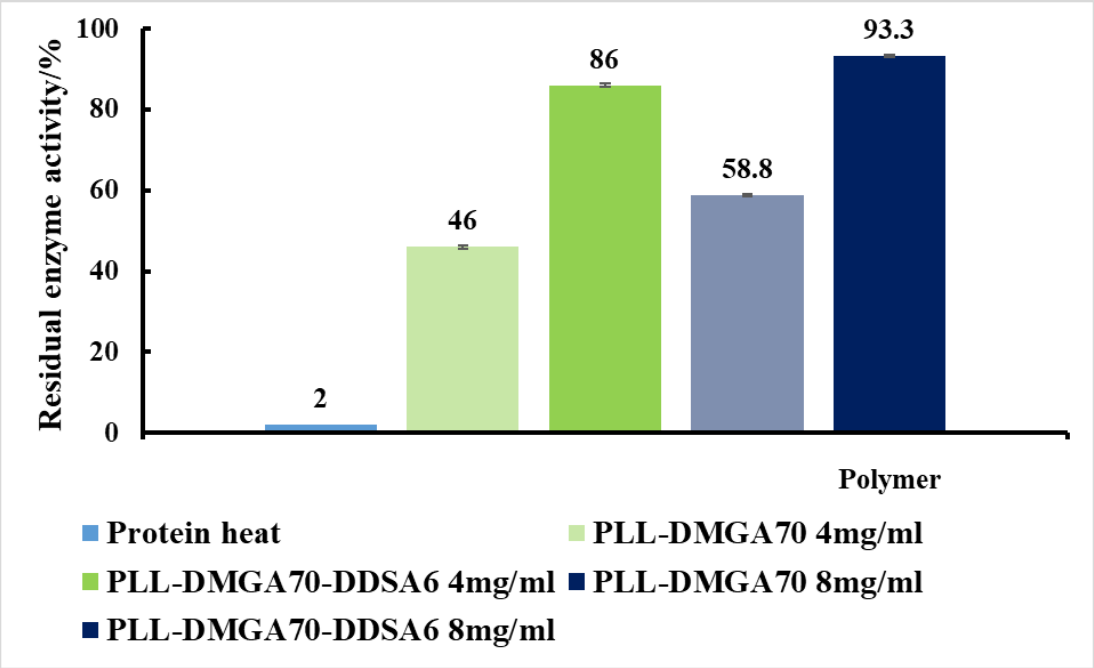


Figure 3.15 Schematic of the efficiency of different polymers in inhibiting protein aggregation at different concentrations

The polymer shown in the figure shows remarkable efficiency in inhibiting protein aggregation. Of note, the introduction of high percentages of anhydride SA (85) and DMGA (85), together with the addition of hydrophobic groups, further enhanced the efficiency of this polymer in inhibiting protein aggregation. The synergistic effect of these components not only enhances the overall performance of the polymer, but also enables it to maintain excellent inhibition at low concentrations.

Firstly, the introduction of anhydrides significantly enhances the charge density of the polymer, which allows the polymer to interact more efficiently with the protein surface, thereby preventing the aggregation of protein molecules.[65] Secondly, the addition of hydrophobic groups further stabilized the inhibitory effect by increasing the hydrophobic interaction between the polymer and the protein. This dual mechanism of action allows the polymer to still exert strong inhibitory ability at low concentrations.

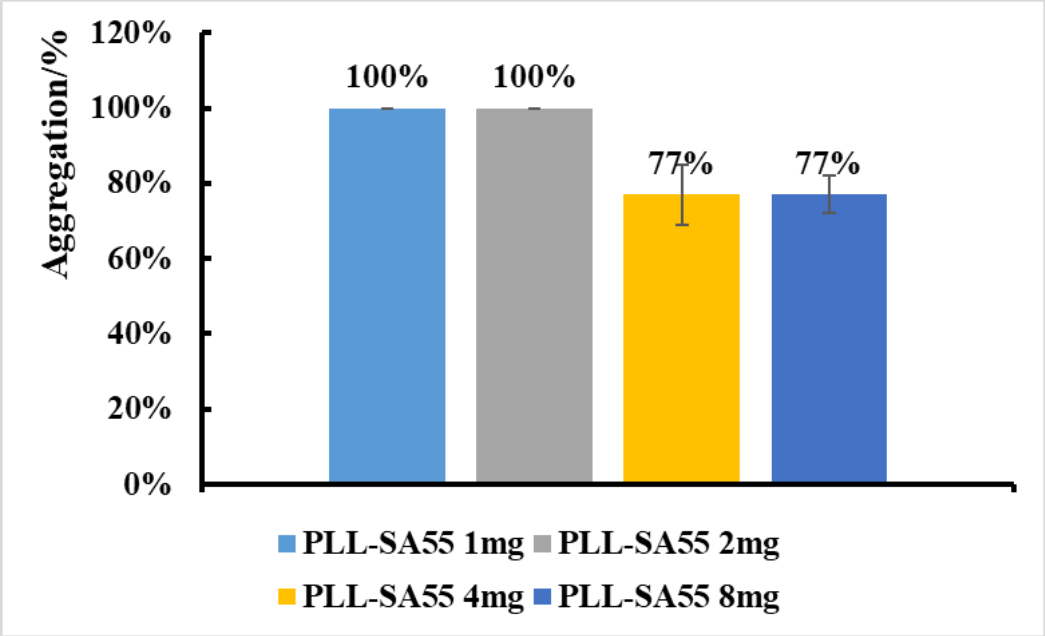
Furthermore, experimental data demonstrate that the polymer significantly reduces the incidence of protein aggregation even at low polymer concentrations, while maintaining the biological activity and functionality of the protein. This property makes the polymer promising for a wide range of applications in biomedicine, food processing, and materials science.

3.6.6 Thioflavin T assay

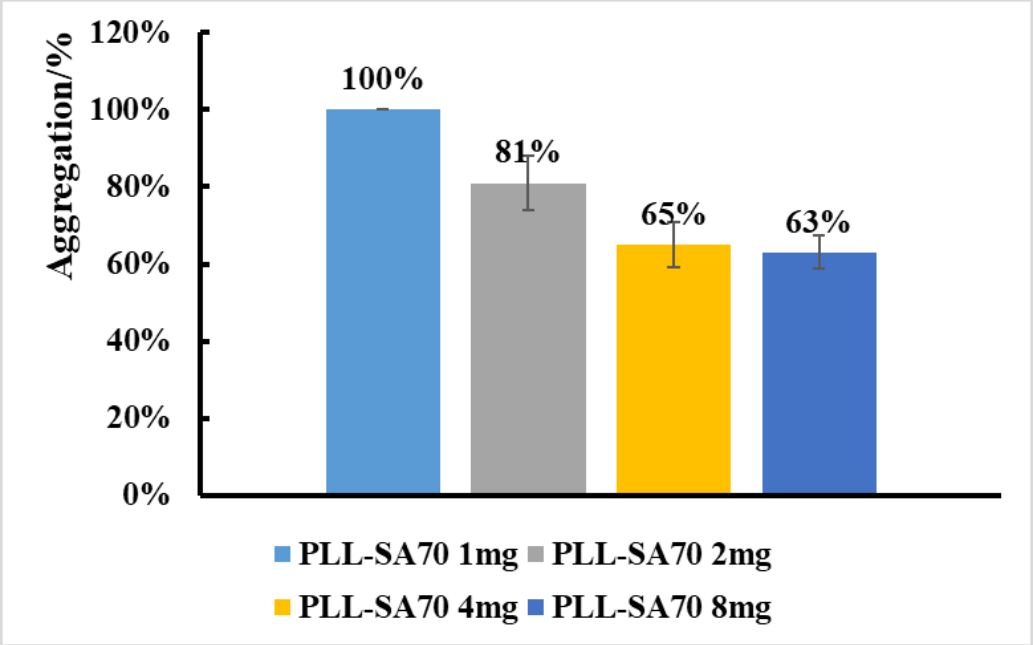
ThT (thioflavin T), as a fluorescent dye, plays an important role in detecting amyloid aggregation. It significantly enhances fluorescence intensity by forming complexes with amyloid fibers, thereby enabling the quantitative detection of the degree of amyloid aggregation.[66] The core principle of ThT detection is the unique β-folded sheet structure of amyloid fibers, which can significantly enhance the fluorescence signal of ThT and provide a precise quantitative method for amyloid aggregation.

All the experimental results are shown in Figure. 3.16 (a) (b) (c) (d) (e) (f).

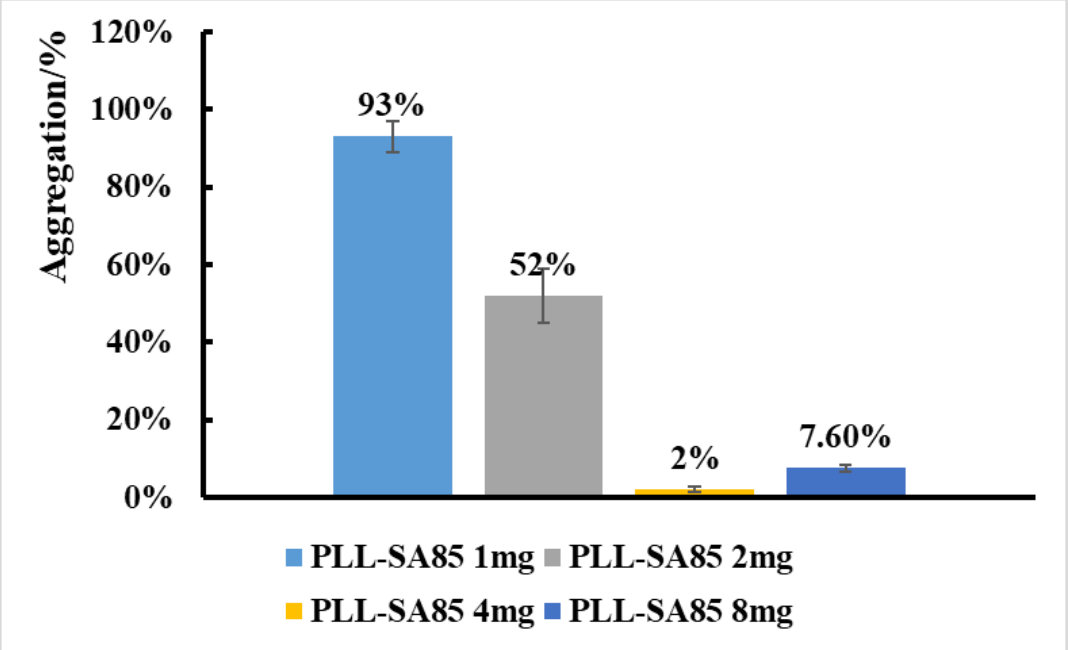
a



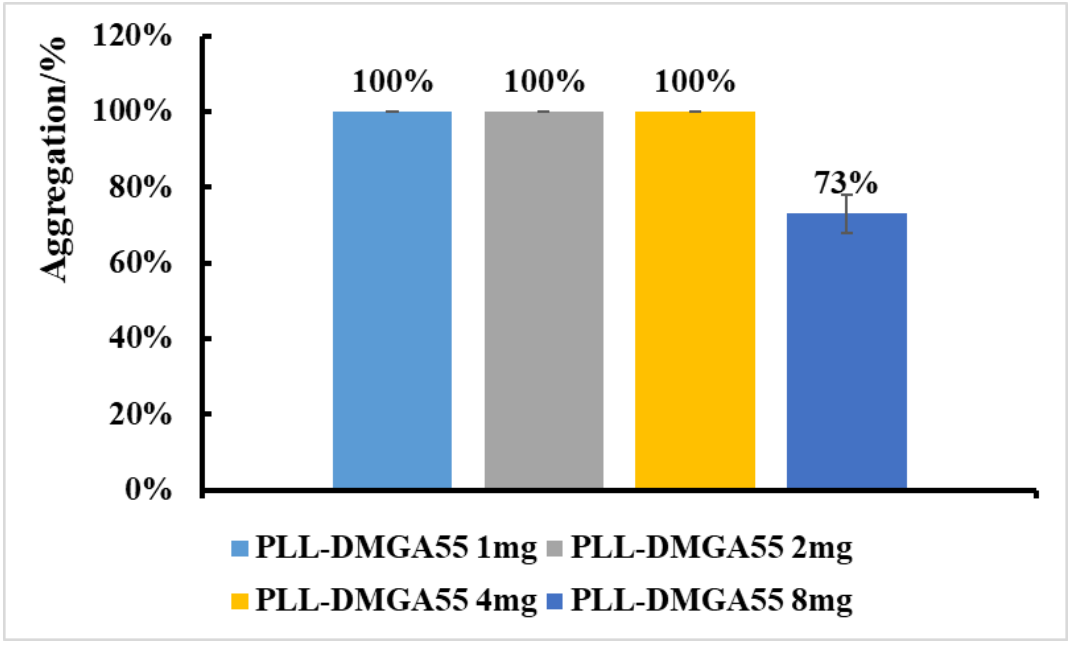
b


Aggregation/%
120%
100%
80%
60%
40%
20%
0%
100%
81%
65%
63%
PLL-SA70 1mg
PLL-SA70 2mg
PLL-SA70 4mg
PLL-SA70 8mg

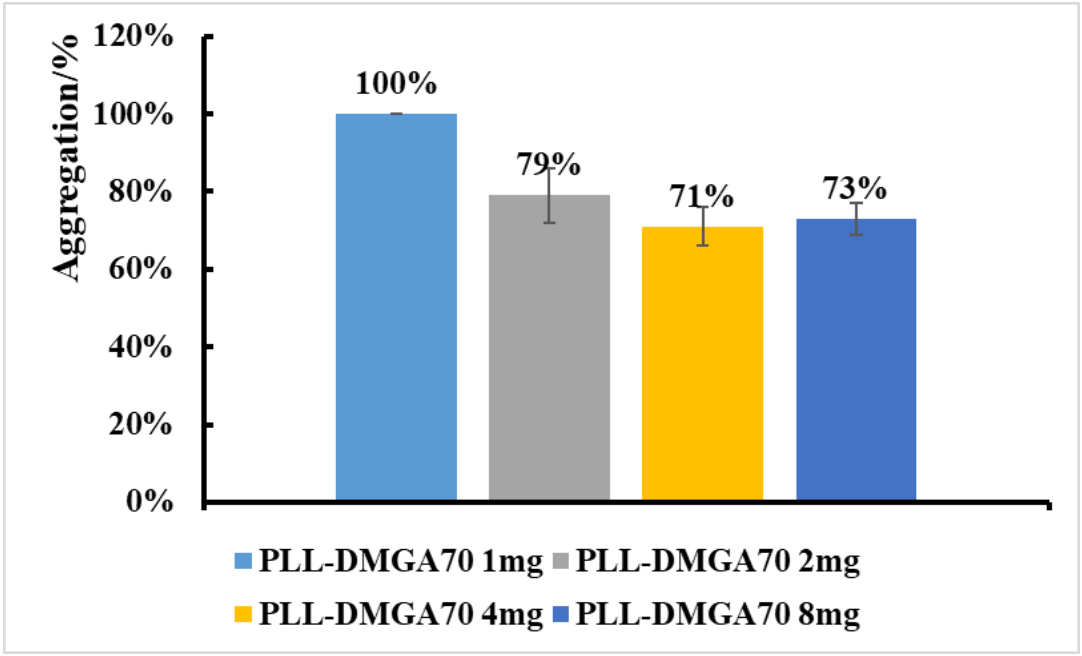
c


Aggregation/%
120%
100%
80%
60%
40%
20%
0%
93%
52%
2%
7.60%
PLL-SA85 1mg
PLL-SA85 2mg
PLL-SA85 4mg
PLL-SA85 8mg

d


Aggregation/%
120%
100%
80%
60%
40%
20%
0%
100%
100%
100%
73%
PLL-DMGA55 1mg
PLL-DMGA55 2mg
PLL-DMGA55 4mg
PLL-DMGA55 8mg

e


Aggregation/%
120%
100%
80%
60%
40%
20%
0%
100%
79%
71%
73%
PLL-DMGA70 1mg
PLL-DMGA70 2mg
PLL-DMGA70 4mg
PLL-DMGA70 8mg

f

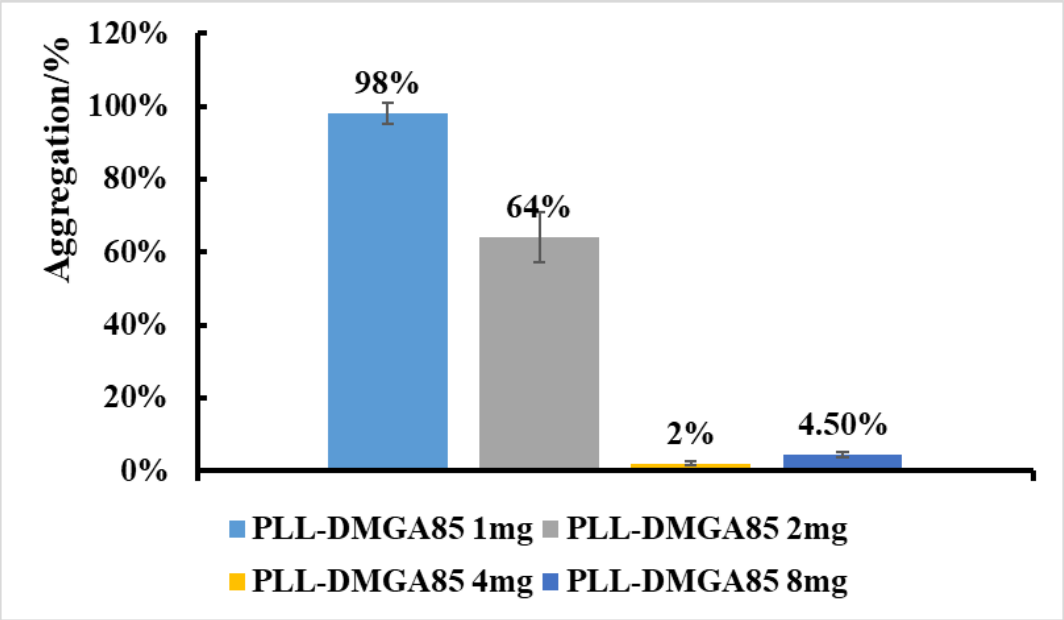


Figure 3.16 (a) (b) (c) (d) (e) (f) The degree of aggregation of proteins after different concentrations of polymers are mixed with proteins and heated.

It can be clearly observed from the figure that with the gradual increase in the percentage of anhydride contained in the polymer in the sample, the aggregation phenomenon in the protein solution is significantly reduced. In particular, in two polymers, PLL-SA (85) and PLL-DMGA (85), protein aggregation was almost completely inhibited. In addition, I found that the polymer maintained its potent inhibitory effect even at lower concentrations.

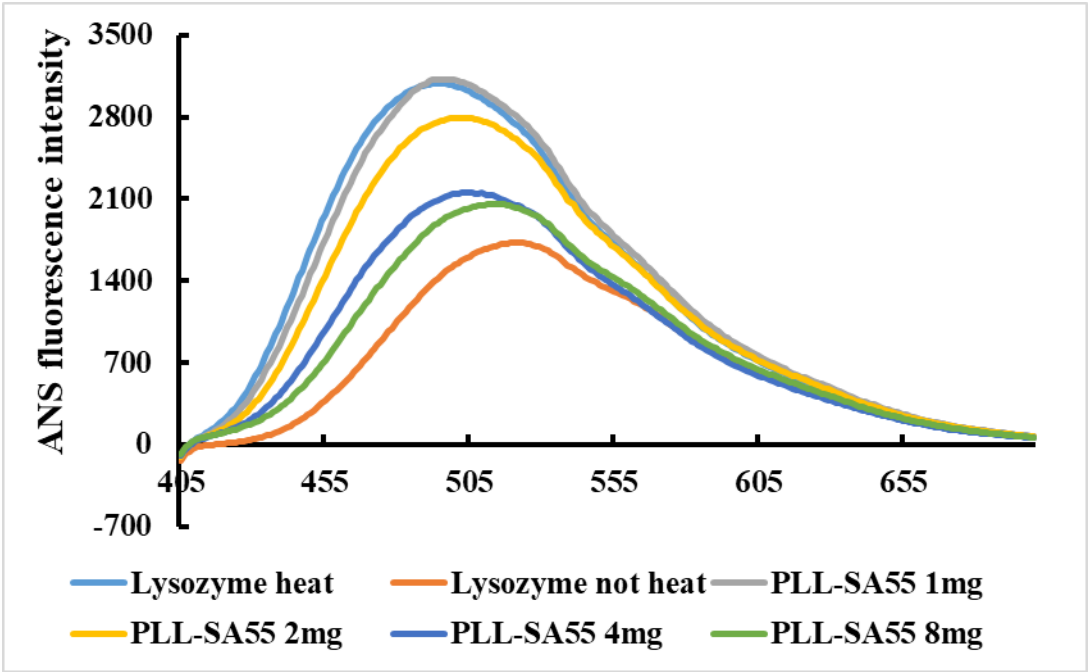
3.6.7 ANS Assay

ANS Assay is a widely used fluorescent probe technique specifically designed to probe the aggregation state of proteins. ANS, as a hydrophobic fluorescent dye, can emit a strong fluorescent signal when bound to proteins.[67] By precise monitoring changes in fluorescence intensity, researchers can indirectly assess the degree of protein aggregation.

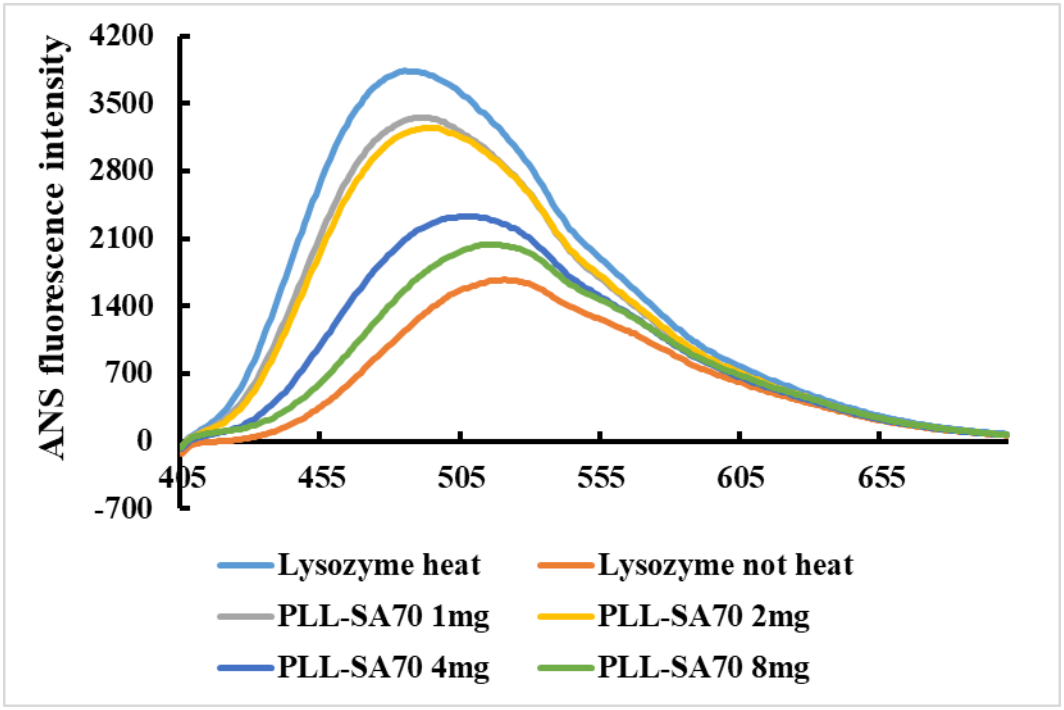
ANS dyes tend to bind tightly to hydrophobic regions of proteins. When the protein is in a monomeric state, these hydrophobic regions are usually wrapped inside the protein, resulting in difficulty for ANS to bind to it, and thus the fluorescence signal is weak. However, when protein aggregation occurs, hydrophobic regions are exposed, allowing ANS to bind to these regions more efficiently, which in turn significantly enhances the fluorescence signal.

All the experimental results are shown in Figure. 3.17 (a) (b) (c) (d) (e) (f).

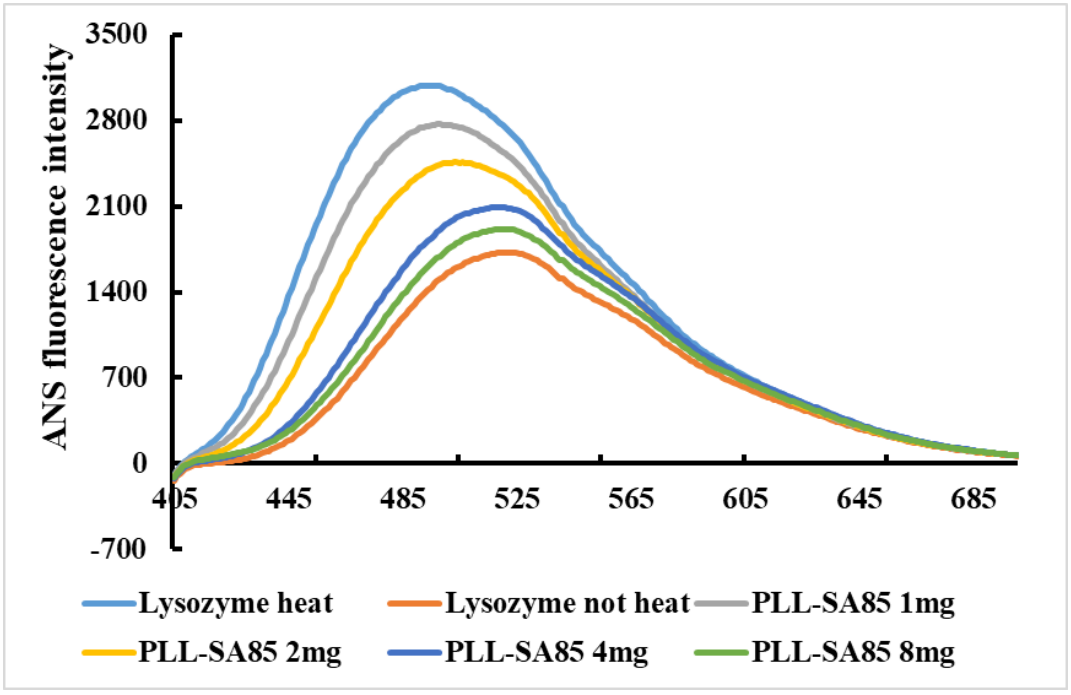
a



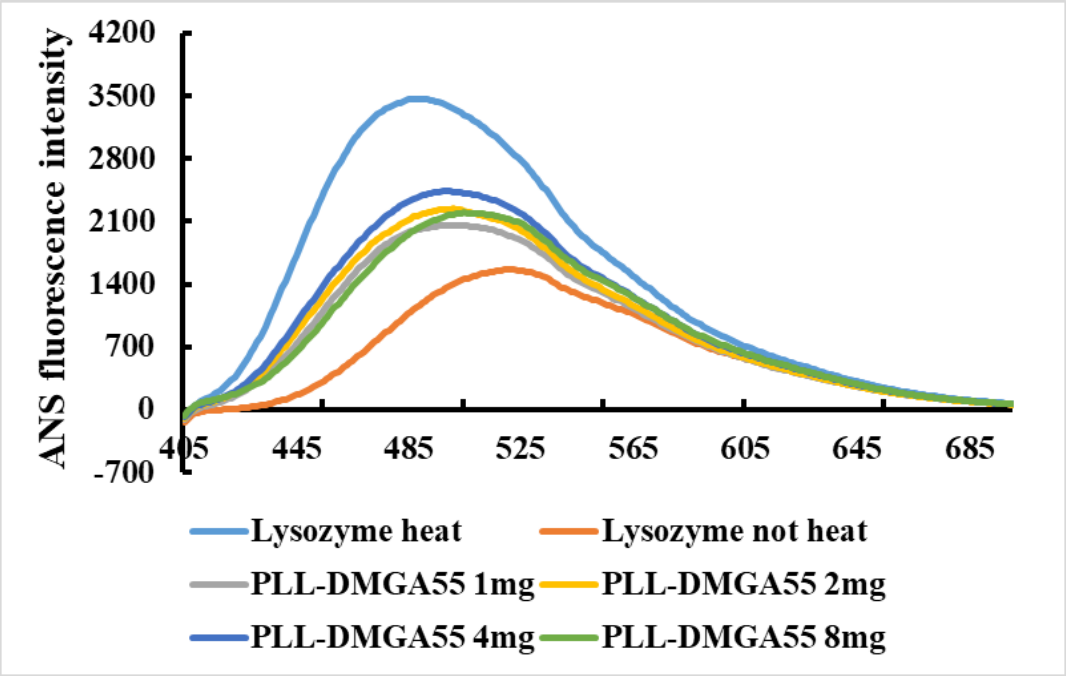
b


ANS fluorescence intensity
4200
3500
2800
2100
1400
700
0
-700
405
455
505
555
605
655
Lysozyme heat
Lysozyme not heat
PLL-SA70 1mg
PLL-SA70 2mg
PLL-SA70 4mg
PLL-SA70 8mg

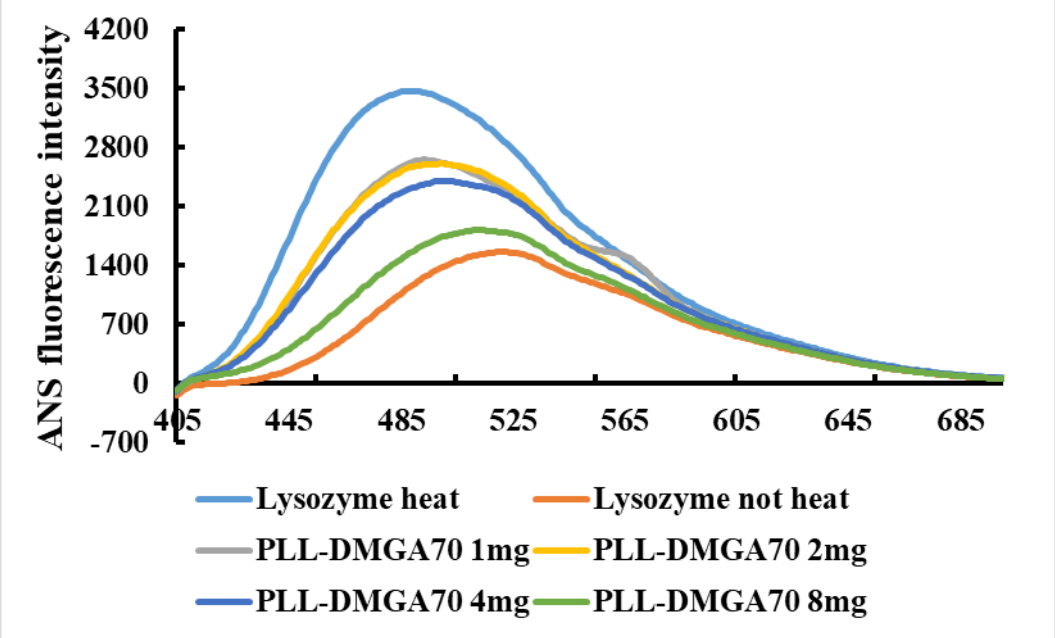
c


ANS fluorescence intensity
3500
2800
2100
1400
700
0
-700
405
445
485
525
565
605
645
685
Lysozyme heat
Lysozyme not heat
PLL-SA85 1mg
PLL-SA85 2mg
PLL-SA85 4mg
PLL-SA85 8mg

d


ANS fluorescence intensity
4200
3500
2800
2100
1400
700
0
-700
405
445
485
525
565
605
645
685
Lysozyme heat
Lysozyme not heat
PLL-DMGA55 1mg
PLL-DMGA55 2mg
PLL-DMGA55 4mg
PLL-DMGA55 8mg

e


ANS fluorescence intensity
4200
3500
2800
2100
1400
700
0
-700
405
445
485
525
565
605
645
685
Lysozyme heat
Lysozyme not heat
PLL-DMGA70 1mg
PLL-DMGA70 2mg
PLL-DMGA70 4mg
PLL-DMGA70 8mg

e

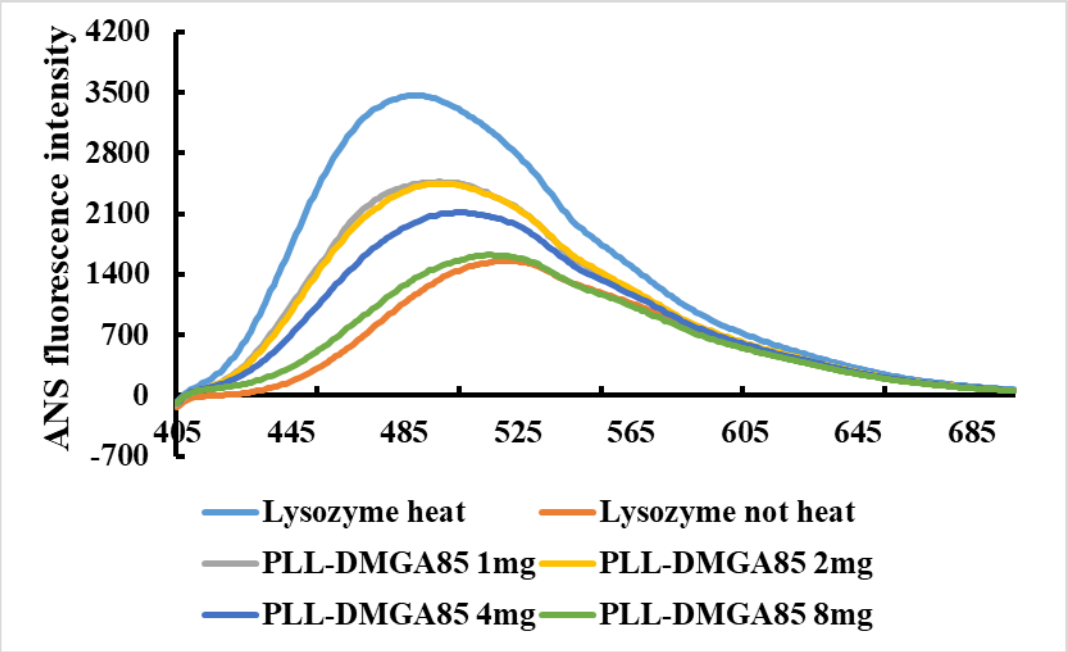


Figure 3.17 (a) (b) (c) (d) (e) (f) ANS fluorescence intensity of proteins after different concentrations of polymers are mixed with proteins and heated.

It can be clearly observed from the figure that when the protein is heated, the hydrophobic part of it binds to the ANS due to exposure, which significantly improves fluorescence intensity. In contrast, unheated proteins did not show this phenomenon. Further experimental results showed that the fluorescence intensity was significantly inhibited after the addition of the polymer, indicating that the presence of the polymer effectively inhibited the structural change of the protein, so that the hydrophobic part of the protein was kept inside the protein. In addition, with the increase of the percentage of acid anhydride contained in the polymer, the fluorescence intensity decreased significantly. In PLL-SA (85) and PLL-DMGA (85) samples, fluorescence was almost completely suppressed by the polymer. At the same time, I found that the polymer was able to maintain its highly effective inhibition even at low concentrations.

3.7 Conclusion

In this part of the experiment, First, I successfully synthesized DMGA and confirmed its high similarity to SA through detailed chemical structure characterization. On this basis, I systematically observed and recorded the dynamic changes of protein aggregation state by reacting DMGA with target proteins under a series of carefully designed experimental conditions. To ensure the accuracy and reliability of the experimental results, I specially set SA as the control group. Through comparative analysis of the data, the remarkable effect of this kind of charged polymer on inhibiting protein aggregation was verified.

Subsequently, I systematically tested the inhibition efficiency of this polymer at different concentrations, and the results showed that this polymer can maintain excellent inhibition effects even at very low concentrations.

Further, I increased the proportion of acid anhydride in the polymer, and the experimental results showed that the polymer with high acid anhydride content was more effective in inhibiting protein aggregation. This phenomenon may be attributed to an increase in the surface charge density of the polymer, which enhances the electrostatic interaction between the protein and the polymer, ultimately inhibiting the aggregation of the protein.

Finally, I optimized the ratio of anhydrides to hydrophobic groups, further improving the efficiency of this polymer in inhibiting protein aggregation.

Chapter 4

Mechanistic insights of the protein protection by polyampholytes

4.1 Introduction

In previous work, I have successfully synthesized a class of charged polymers that show significant potential for inhibiting protein aggregation. Through a series of tests, I comprehensively evaluated the performance of these polymers under different conditions. First, the chemical structure of these charged polymers was examined in detail. Subsequently, I used dynamic light scattering (DLS), residual enzyme activity detection, ThT detection and ANS detection to observe the interaction between the polymer and protein in detail. These experimental results consistently show that charged polymers can effectively interfere with the protein aggregation process and significantly reduce the formation of protein aggregates.

Although I have successfully synthesized these polymers, the ways by which they inhibit protein aggregation have not been fully elucidated. Therefore, I will further explore the specific mechanism of polymer inhibition of protein aggregation through the following experiments.

4.2 Explore ways to inhibit protein aggregation

4.2.1 Protection of protein secondary structure

Protein aggregation refers to the phenomenon that individual protein molecules form polymers or aggregates through non-covalent interactions, such as hydrogen bonding, hydrophobic interactions and van der Waals forces. This aggregation process can be either reversible or irreversible. During the process of protein aggregation, its secondary structure also changes significantly. The secondary structure of a protein is a locally stable structure maintained by hydrogen bonds, mainly including α-helix and β-folding, which play a decisive role in the three-dimensional folding process of a protein and directly affect the overall conformation and function of the protein. Therefore, changes in the secondary structure during protein aggregation can fundamentally alter the properties of the protein, causing it to lose its intended function. Therefore, in the process of inhibiting protein aggregation, it is particularly important to maintain the stability of secondary structure.

In the secondary structure of proteins, folded proteins exhibit a wide range of chemical shifts in NMR spectra due to the anisotropic magnetic fields of aromatic or carbonyl groups.[116] In contrast, the amino acid peaks of denatured proteins are not obvious due to their random helical conformation, resulting in a relatively narrow range of chemical shifts.[68]

Based on this, in the experiment, I will examine the chemical shifts of some secondary structures in the protein by nuclear magnetic resonance and how these chemical shifts change after heating. The experimental results are shown in Figure 4.1

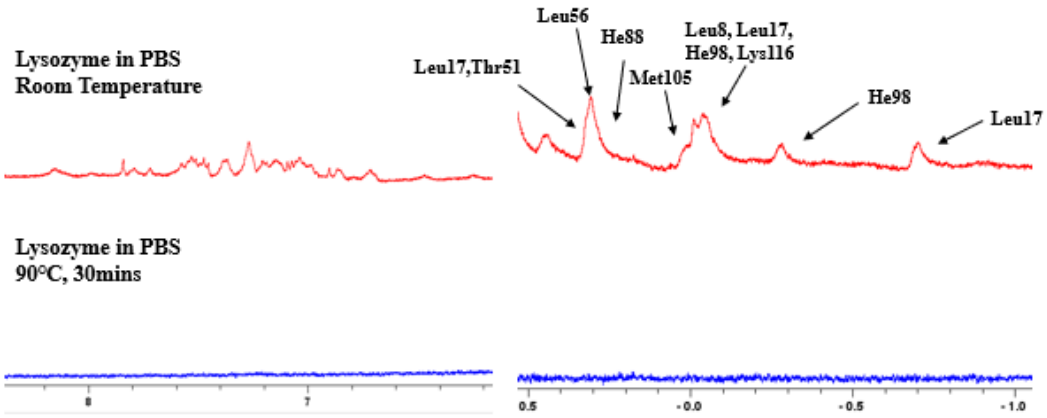


Figure 4.1 ^{1}H NMR spectra of the lysozyme at room temperature and after heating to 90 °C for 30 min.

As shown in figure 4.1, the unheated protein exhibits a wide range of chemical shift signals in the plot. And after heating to 90°C and lasting for 30 minutes, I can observe that all these signals disappear. This experimental result indicates that the secondary structure of lysozyme is destroyed after heating and lysozyme is in a random coil state.

In the next experiment, the chemical shift of part of the secondary structure of the protein solution which was added to the polymer and heated for 30 minutes was detected. The experimental results are shown in Figure. 4.2 and Figure. 4.3.

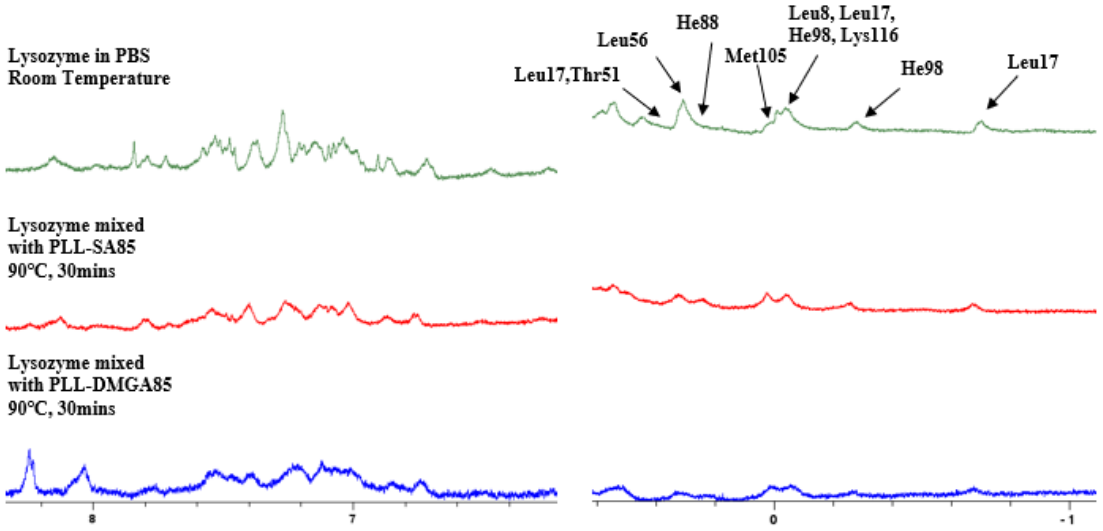


Figure 4.2 ^{1}H NMR spectra of the lysozyme at room temperature and the lysozyme mixed with polymer (8mg/ml) after heated.

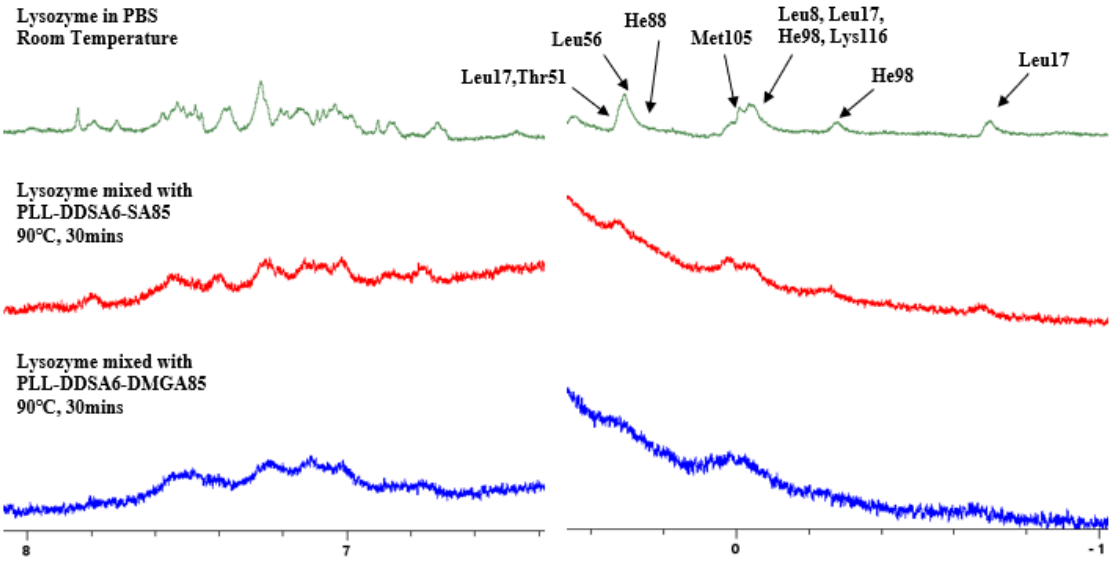


Figure 4.3 ^{1}H NMR spectra of the lysozyme at room temperature and the lysozyme mixed with polymer(8mg/ml) after heated.

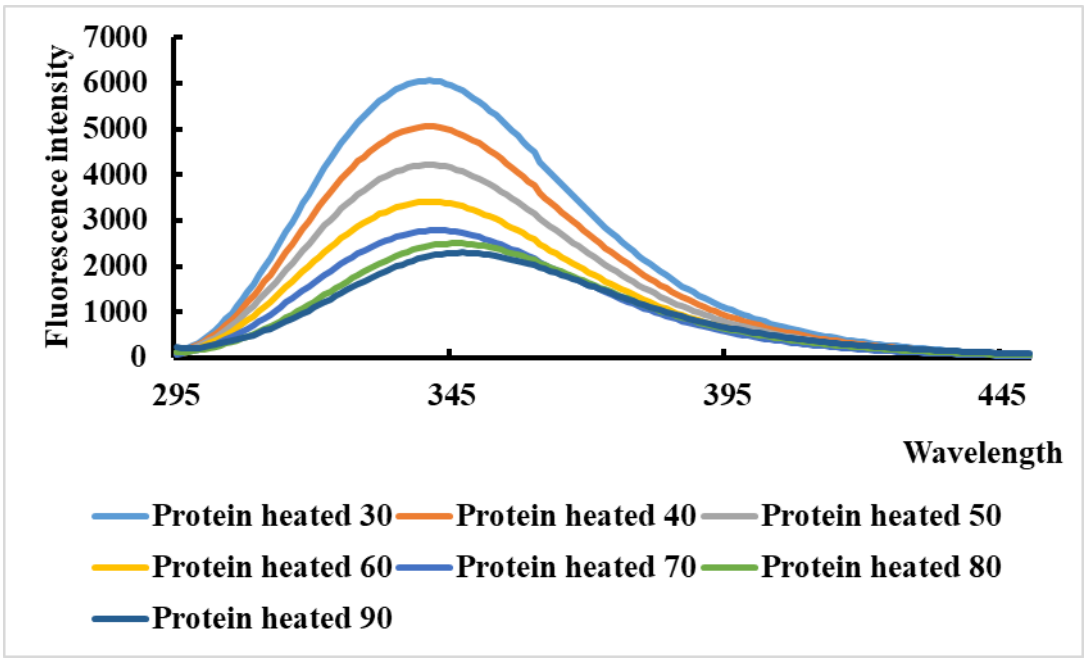
It can be clearly observed from the figure that in the presence of the polymer, most of the chemical shift signal of lysozyme is still maintained after heating treatment. This phenomenon suggests that lysozyme can retain most of its secondary structure and maintain the integrity of some higher-order structures.

According to previous reports, most of the peaks observed in NMR profiles can be attributed to specific amino acid residues in the secondary structure of proteins. [69] Therefore, these experimental results strongly demonstrate that the polymer can effectively protect the secondary structure of lysozyme and some higher-order structures, thus significantly improving the stability of the protein at high temperatures. This is further confirmed by the distribution of signals in the forefield region, which mainly corresponds to amino acid residues in secondary structures such as α-helical, β-lamellar and ring structures.[70,71]

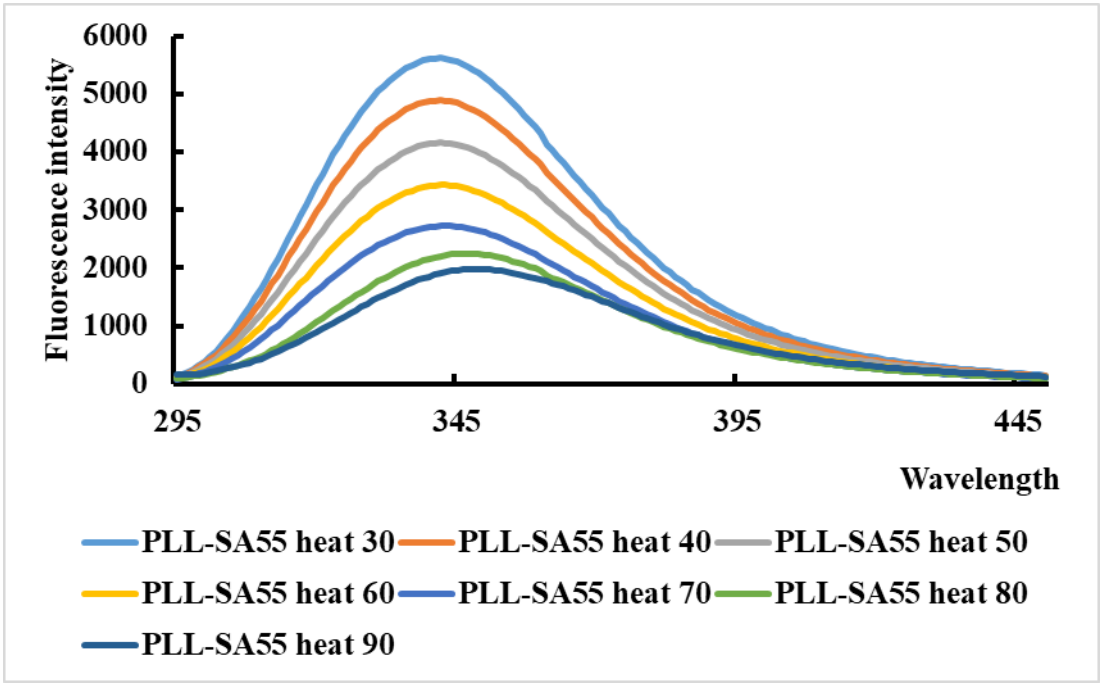
4.2.2 Phase diagrams during lysozyme aggregation as detected by fluorescence

Before showing the phase diagram, I first need to measure the fluorescence changes of proteins at different temperatures, Studies have shown that Trp-62 and Trp-108 are the main fluorophore in the lysozyme structure, and they are located at the bottom of the binding region of the lysozyme structure. The change of fluorescence intensity was positively correlated with the activity of protein.[72] The experimental results are shown in Figure 4.3 (a) (b) (c) (d) (e) (f) (g) (h) (i).

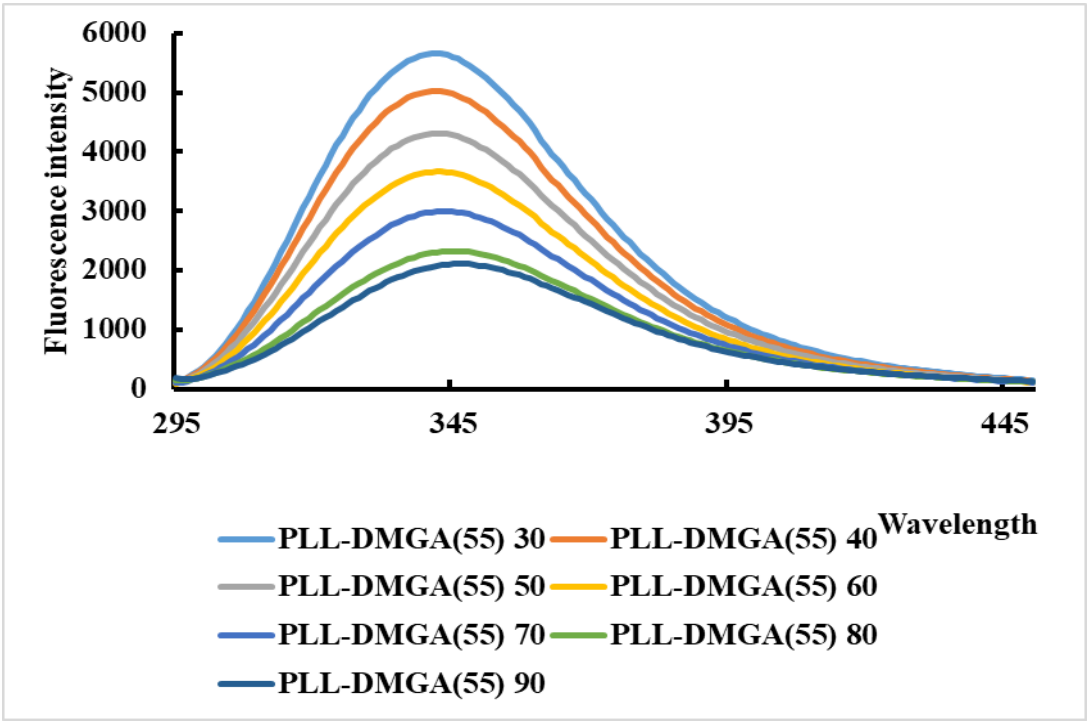
a



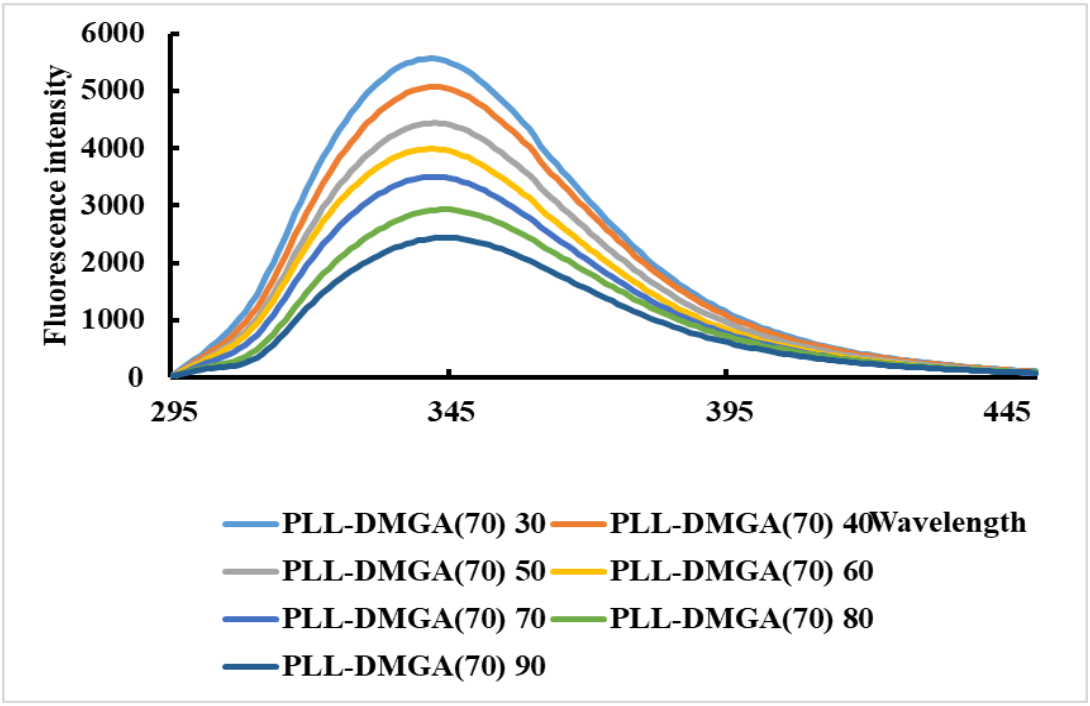
b


Fluorescence intensity
6000
5000
4000
3000
2000
1000
0
295
345
395
445
Wavelength
PLL-SA55 heat 30
PLL-SA55 heat 40
PLL-SA55 heat 50
PLL-SA55 heat 60
PLL-SA55 heat 70
PLL-SA55 heat 80
PLL-SA55 heat 90

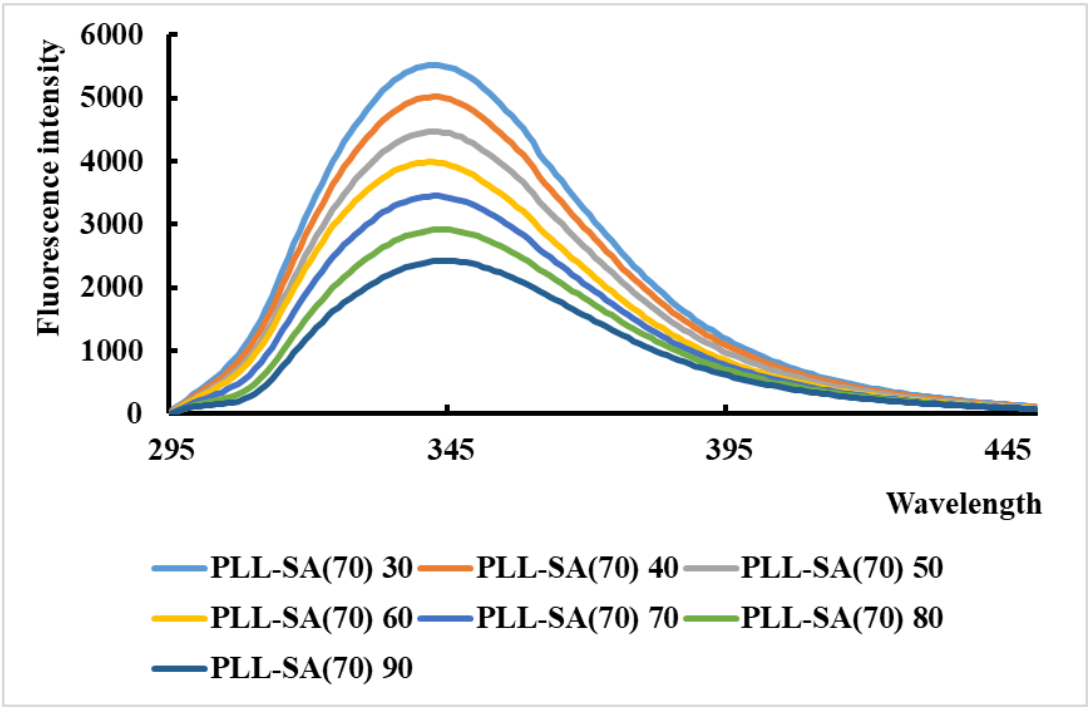
c


Fluorescence intensity
6000
5000
4000
3000
2000
1000
0
295
345
395
445
Wavelength
PLL-DMGA(55) 30
PLL-DMGA(55) 40
PLL-DMGA(55) 50
PLL-DMGA(55) 60
PLL-DMGA(55) 70
PLL-DMGA(55) 80
PLL-DMGA(55) 90

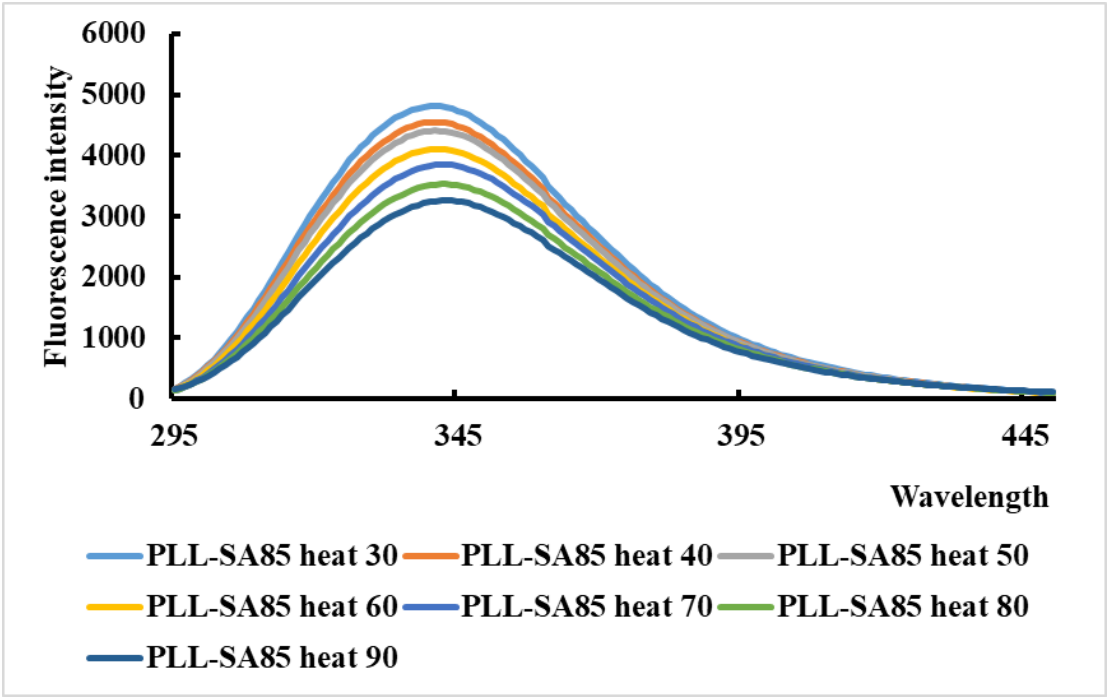
d


6000
5000
4000
3000
2000
1000
0
Fluorescence intensity
295
345
395
445
Wavelength
PLL-DMGA(70) 30
PLL-DMGA(70) 40
PLL-DMGA(70) 50
PLL-DMGA(70) 60
PLL-DMGA(70) 70
PLL-DMGA(70) 80
PLL-DMGA(70) 90

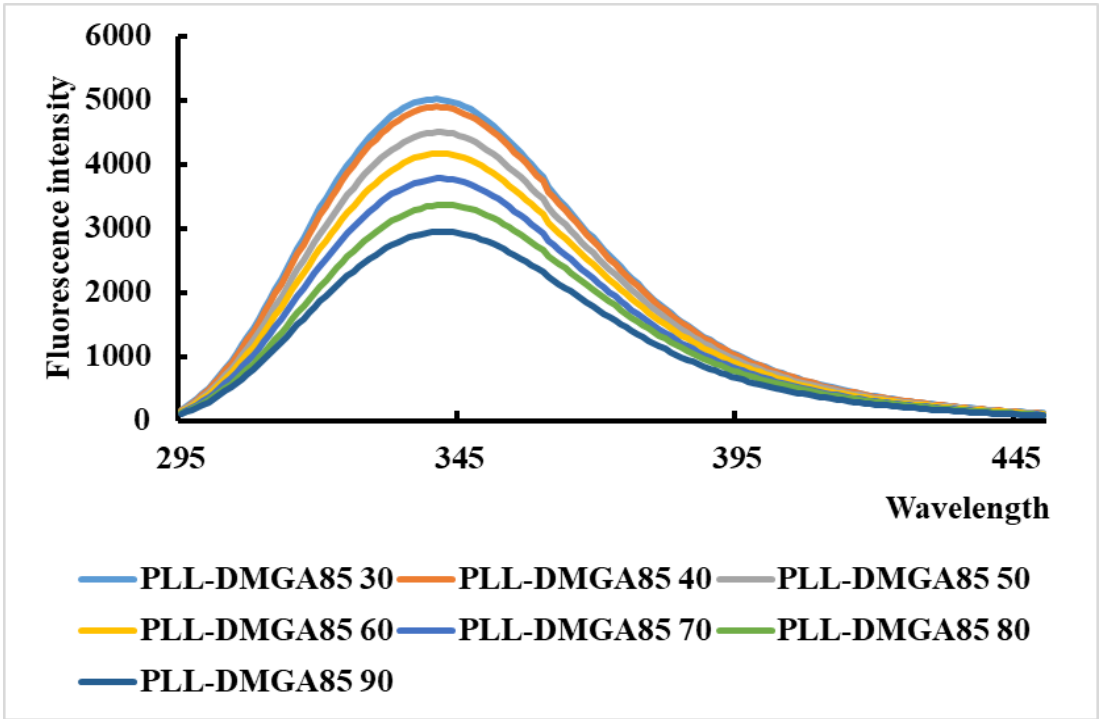
e


6000
5000
4000
3000
2000
1000
0
Fluorescence intensity
295
345
395
445
Wavelength
PLL-SA(70) 30
PLL-SA(70) 40
PLL-SA(70) 50
PLL-SA(70) 60
PLL-SA(70) 70
PLL-SA(70) 80
PLL-SA(70) 90

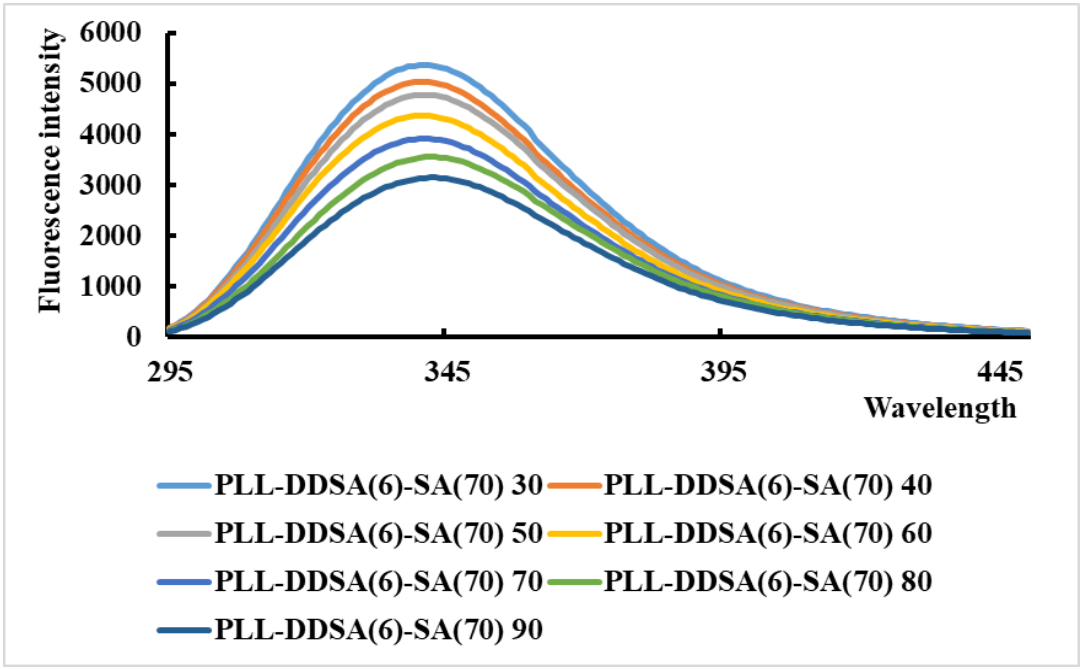
f


6000
5000
4000
3000
2000
1000
0
Fluorescence intensity
295
345
395
445
Wavelength
PLL-SA85 heat 30
PLL-SA85 heat 40
PLL-SA85 heat 50
PLL-SA85 heat 60
PLL-SA85 heat 70
PLL-SA85 heat 80
PLL-SA85 heat 90

g


6000
5000
4000
3000
2000
1000
0
Fluorescence intensity
295
345
395
445
Wavelength
PLL-DMGA85 30
PLL-DMGA85 40
PLL-DMGA85 50
PLL-DMGA85 60
PLL-DMGA85 70
PLL-DMGA85 80
PLL-DMGA85 90

h



i

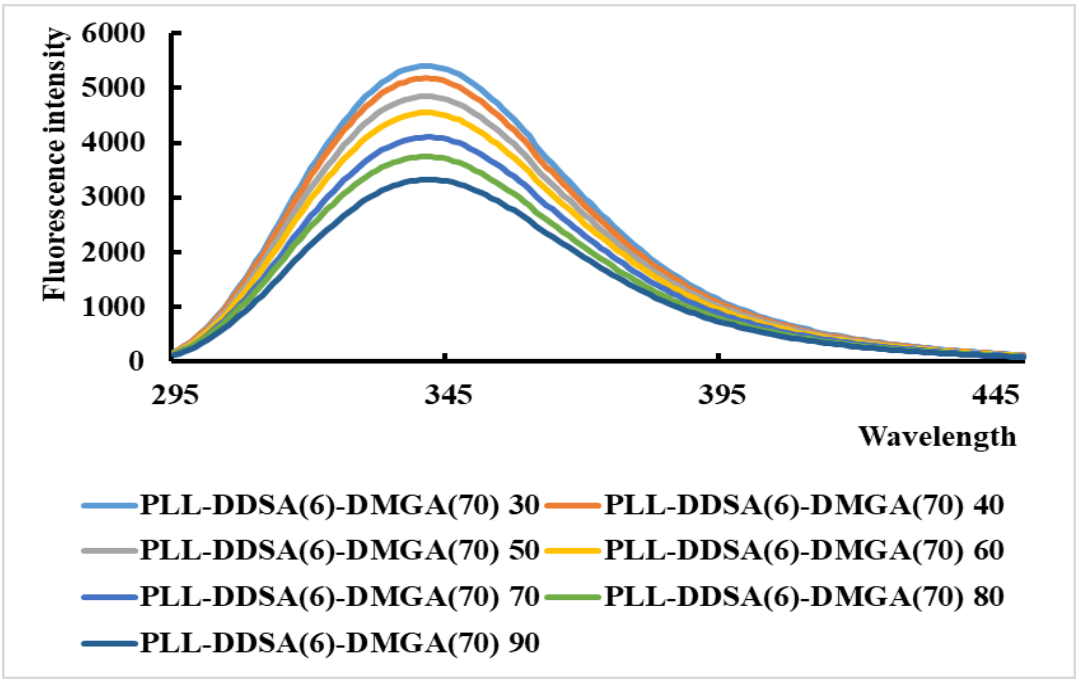


Figure 4.3 (a) (b) (c) (d) (e) (f) (g) (h) (i) Fluorescence changes of proteins and proteins mixed with polymers at different temperatures.

It can be clearly observed from the experimental results that the fluorescence intensity of protein and its mixture with polymer shows a trend of decreasing with the gradual increase of temperature. This phenomenon indicates that with the increase of temperature, some amino acid residues inside the protein undergo structural changes, resulting in the gradual loss of fluorescence activity. At the same time, I also found that the decline rate of fluorescence intensity slowed down significantly after the addition of polymer, which indicates that the addition of polymer can inhibit the structural changes of amino acid residues in proteins to a certain extent.

Protein Fluorescence Phase Diagram is a tool used to study the conformational change and phase transition behavior of proteins under different conditions. The fluorescence intensity of proteins can be monitored under different conditions such as temperature, pH value and ionic strength, to reveal the conformational stability, aggregation state and phase transition process of proteins.

Phase diagrams are often used to analyze the fluorescence data of protein molecules. The core of phase diagram is to reveal the behavior of protein during denaturation by the intrinsic fluorescence emission intensity of protein at a fixed emission wavelength. Specifically, by applying external stresses or additives, I can determine the fluorescence emission intensity I(λ1) and I(λ2) of protein molecules at two different emission wavelengths λ1 and λ2. The combined analysis of these data results in a phase map of the protein molecule.[73]

$$I(\lambda_1) = a + bI(\lambda_2) \quad \text{(Equation 1)}$$

$$\mathrm{a} = I_1(\lambda_1) - \frac{I_2(\lambda_1) - I_1(\lambda_1)}{I_2(\lambda_2) - I_1(\lambda_2)} I_1(\lambda_2) \quad \text{(Equation 2)}$$

$$\mathrm{b} = \frac{I_2(\lambda_1) - I_1((\lambda_1)}{I_2(\lambda_2) - I_1(\lambda_2)} \quad \text{(Equation 3)}$$

When equation 1 is linear, it reveals an all-or-nothing transition between two different conformations, meaning that no partial intermediates are present.[122] However, if equation 1 is nonlinear, each linear paragraph represents a transitional stage, indicating that there may be one or more intermediate stages in the protein folding process.[74]

Based on this, I drew the phase diagram of lysozyme and its mixed polymer under the condition of gradually increasing temperature(30℃-90℃). This phase diagram details the phase transition behavior of lysozyme and its polymers at different temperature gradients. All experimental data were mapped at I330nm and I341nm.

First, it is the phase diagram of the protein without added polymer and gradually increasing the temperature. The experimental results are shown in Figure 4.4

c

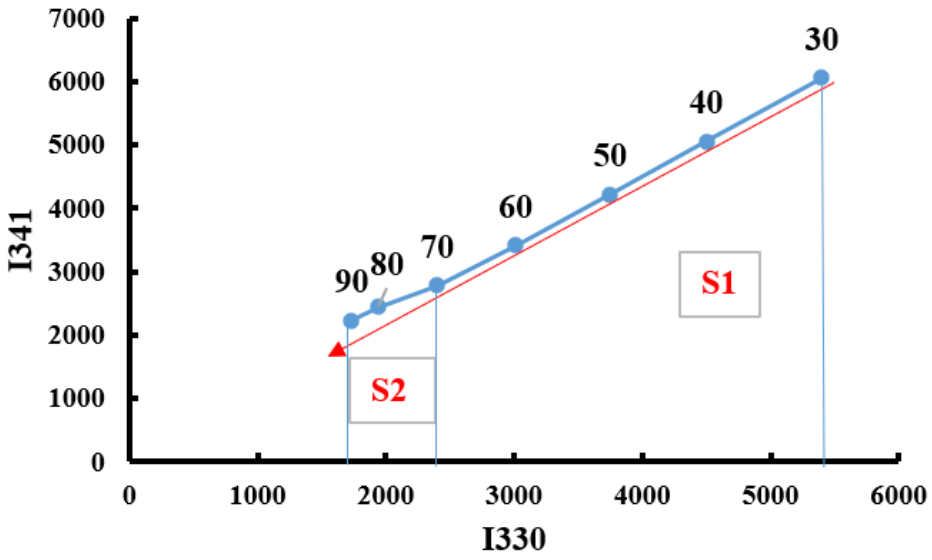


Figure 4.4 The phase diagram of the protein without added polymer and gradually increasing the temperature.

As shown in the figure, without the addition of polymers, two conformational states (S1 and S2) are clearly present in the lysozyme phase diagram. This phenomenon indicates that there is a significant intermediate state in the transition from natural lysozyme to denaturetic lysozyme.

Further analysis revealed that the conformational transition temperature of lysozyme was roughly between 70℃ and 80℃. This result is consistent with the known irreversible denaturation temperature of lysozyme (74℃) and indicates that the protein can undergo irreversible denaturation in the conformational state of S2.

Next, it is the phase diagram of the protein with the polymer added and gradually increasing the temperature. The experimental results are shown in Fig. 4.5 (a) (b) (c) (d)

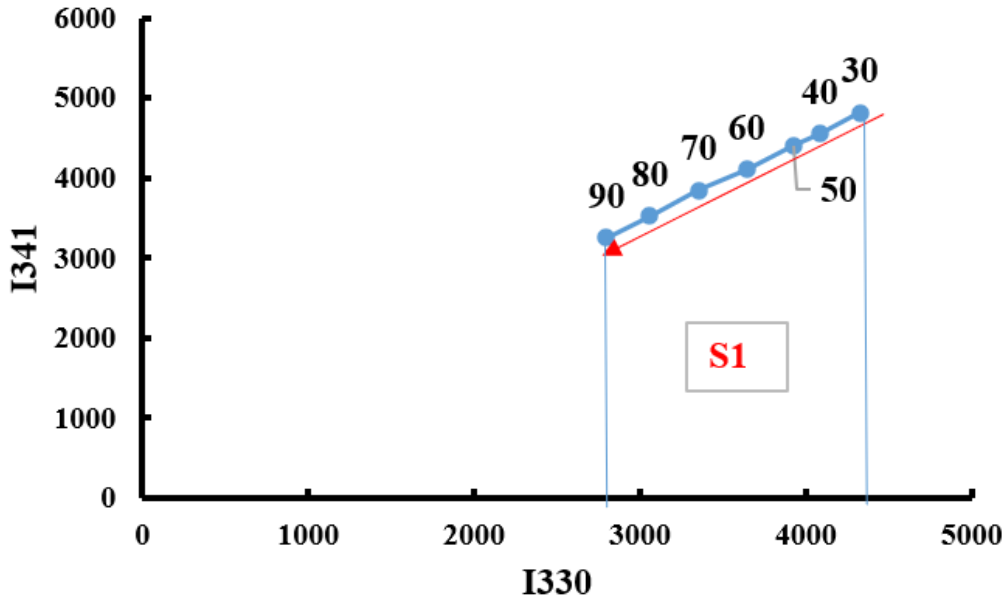


Figure 4.5 a The phase diagram of the protein mixed PLL-SA85 and gradually increasing the temperature.

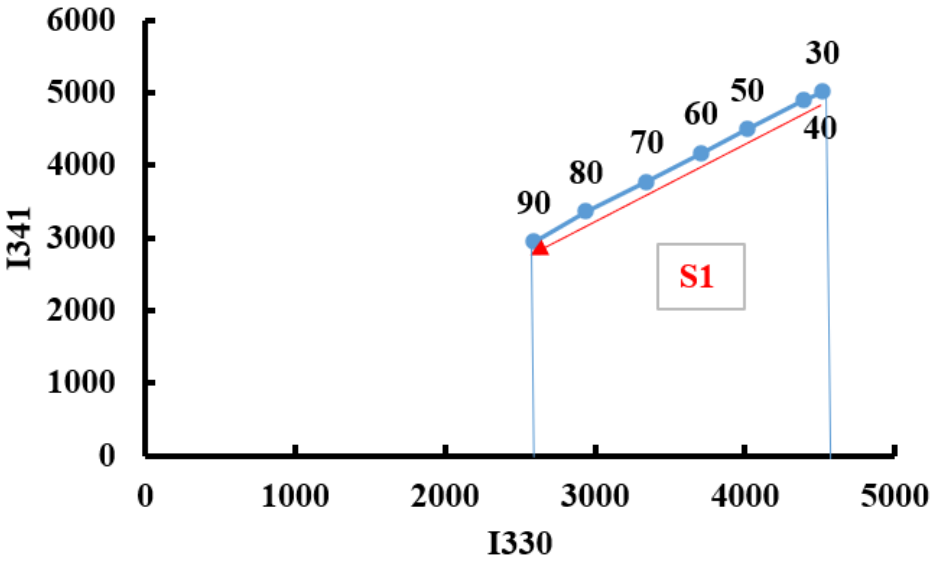


Figure 4.5 b The phase diagram of the protein mixed PLL-DMGA85 and gradually increasing the temperature.

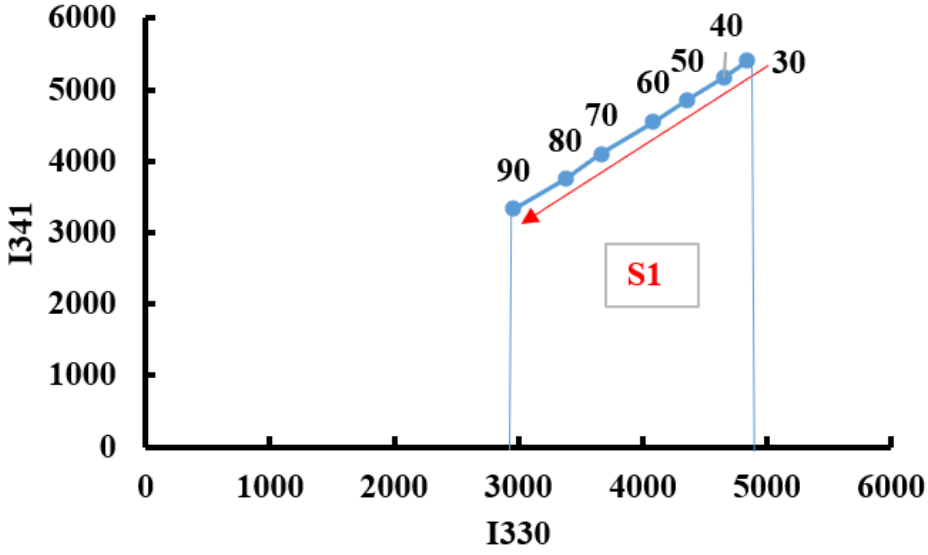


Figure 4.5 c The phase diagram of the protein mixed PLL-DDSA6-DMGA70 and gradually increasing the temperature.

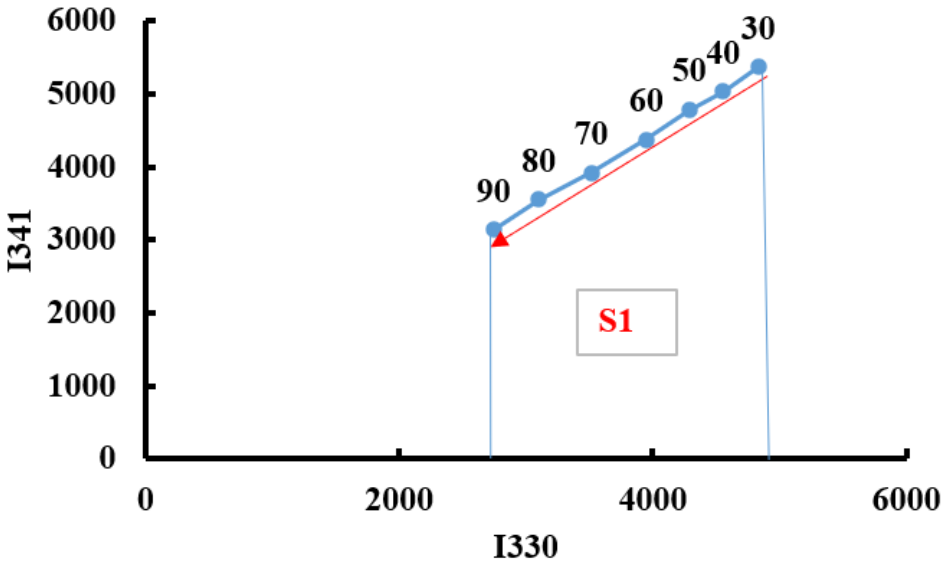


Figure 4.5 d The phase diagram of the protein mixed PLL-DDSA6-SA70 and gradually increasing the temperature.

It can be clearly seen from the experimental results that the slope of the protein phase diagram with the addition of the polymer remains constant during the process of gradual heating under the same temperature conditions. This phenomenon shows that the protein remained in the S1 conformation state throughout the experiment. This not only revealed that the conformational transformation of proteins was inhibited in the presence of polymers, but also further confirmed that the mechanism of polymer inhibition of protein aggregation was to prevent the irreversible conformational transformation of protein molecules.

To verify that lysozyme will not undergo irreversible denaturation in conformation S1, but will undergo irreversible denaturation in conformation S2, I designed and conducted the following comparative tests. First, it is well known that proteins undergo a conformational transformation and undergo denaturation at 74 ° C. Therefore, I conducted protein cycling heating and cooling experiments from 30 ° C to 70 ° C and from 30 ° C to 90 ° C respectively to verify whether irreversible aggregation of proteins does not occur in S1 conformation. Experimental results are shown in Figure. 4.6 a and b.

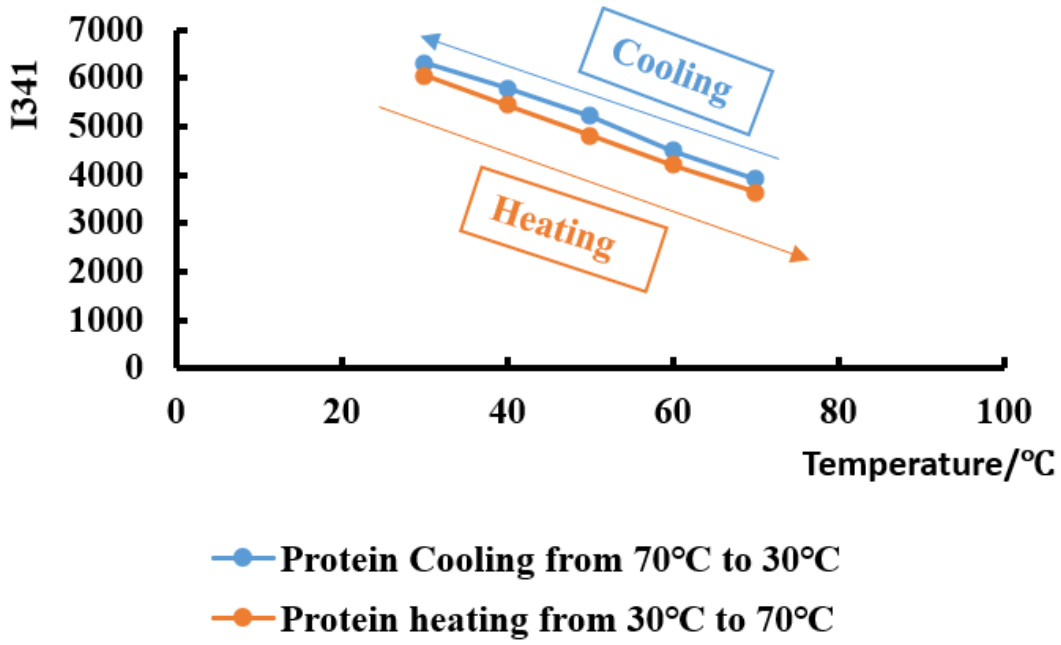


Figure 4.6 a Only protein cycling heating and cooling experiments from 30 ° C to 70 ° C

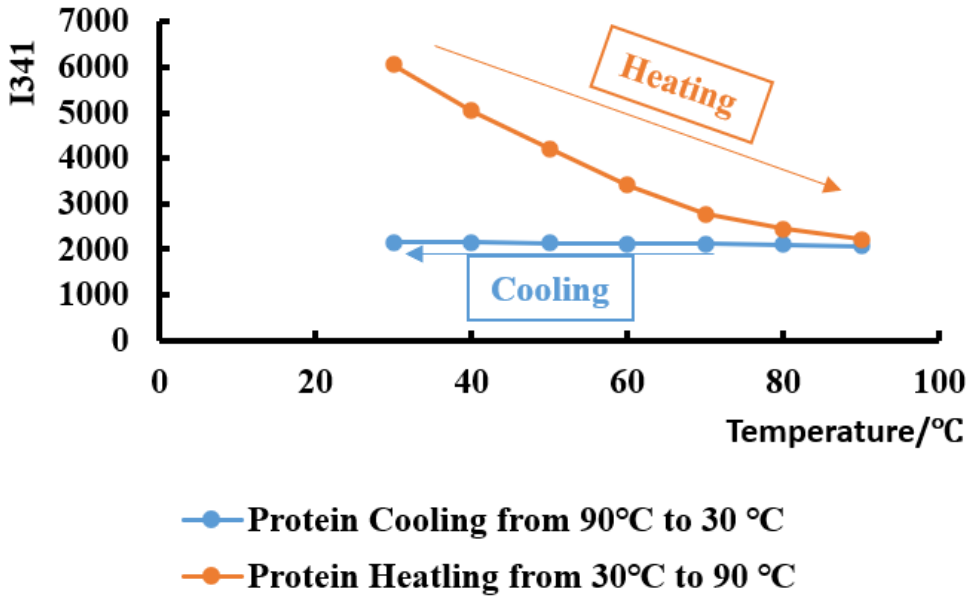
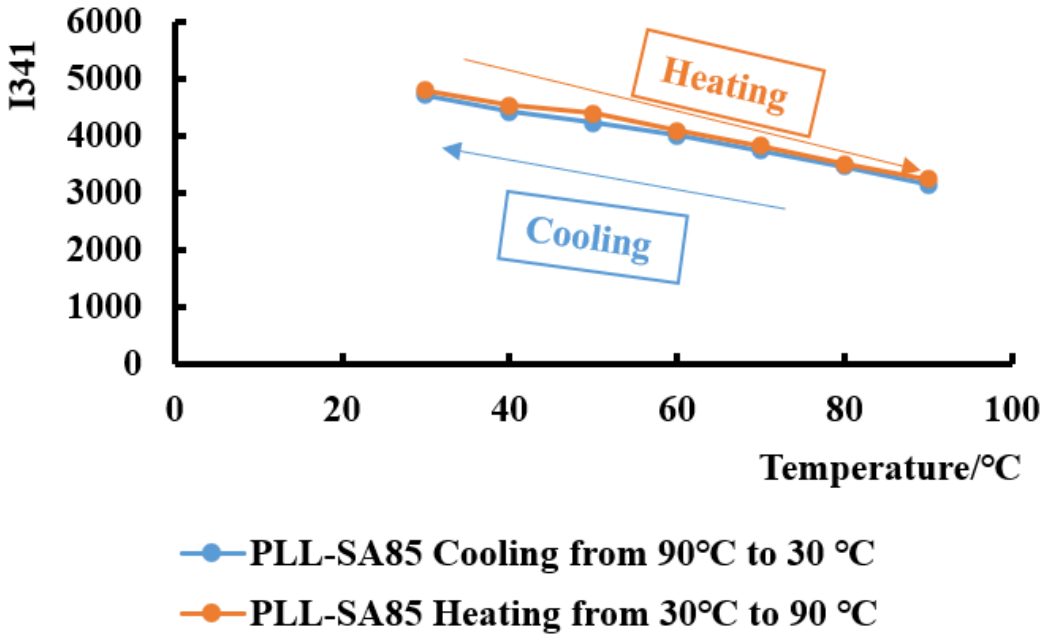


Figure 4.6 b Only protein cycling heating and cooling experiments from 30 ° C to 90 ° C

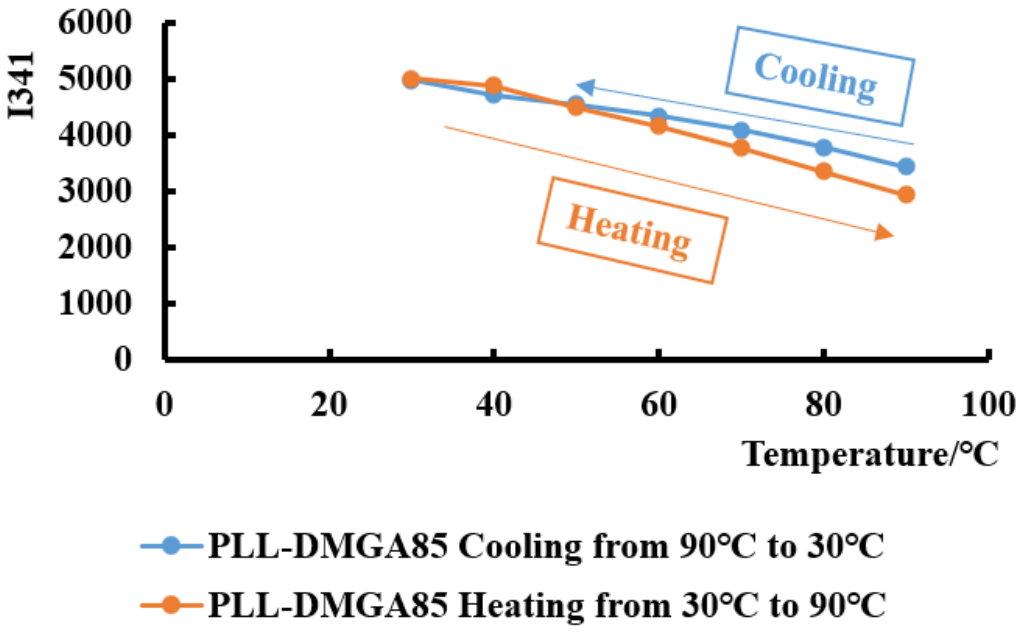
By analyzing the experimental data, I can conclude that when the cyclic heating and cooling experiment is performed in the temperature range of 30 ° C to 70 ° C, the protein is able to refold and return to its natural state after cooling at 70 ° C, which indicates that the conformation (S1) at 70 ° C does not cause irreversible denaturation of the protein. However, when the experimental temperature range was extended to 30℃ to 90℃, it was found that when the protein was in the conformation (S2) at 90℃, its fluorescence intensity could not be recovered with the decrease of temperature, which clearly indicated that the protein in the conformation S2 had undergone irreversible denaturation.

Subsequently, I conducted a cyclic cooling experiment on the protein with added polymer in the range of 30℃ to 90℃, and the results were shown in Figure. 4.7 a b c d.

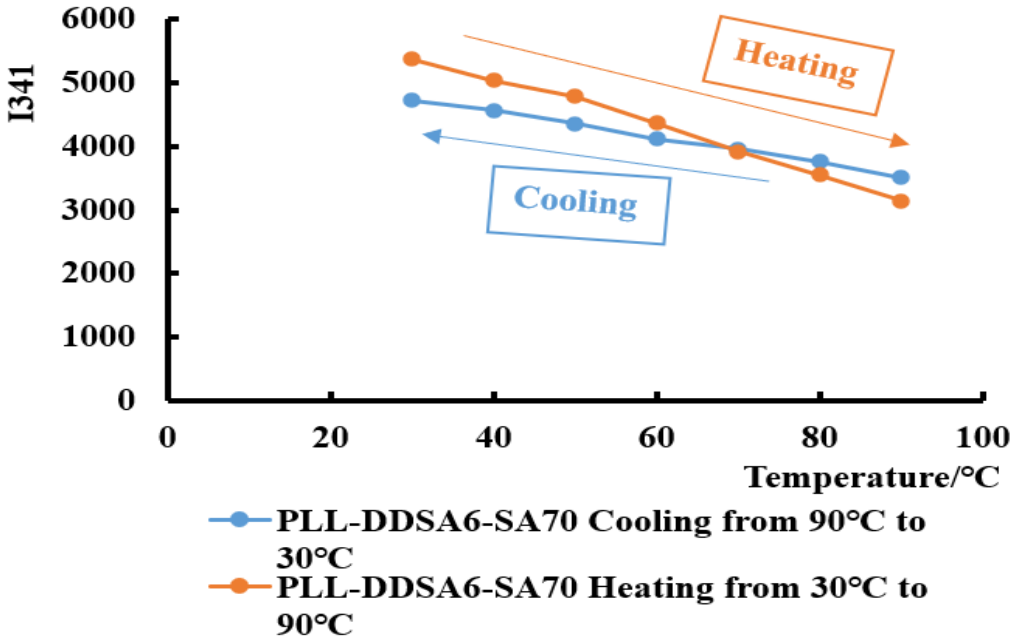
a


I341
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Temperature/°C
Heating
Cooling
PLL-SA85 Cooling from 90°C to 30 °C
PLL-SA85 Heating from 30°C to 90 °C

b


I341
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Temperature/°C
Cooling
Heating
PLL-DMGA85 Cooling from 90°C to 30°C
PLL-DMGA85 Heating from 30°C to 90°C

c



d

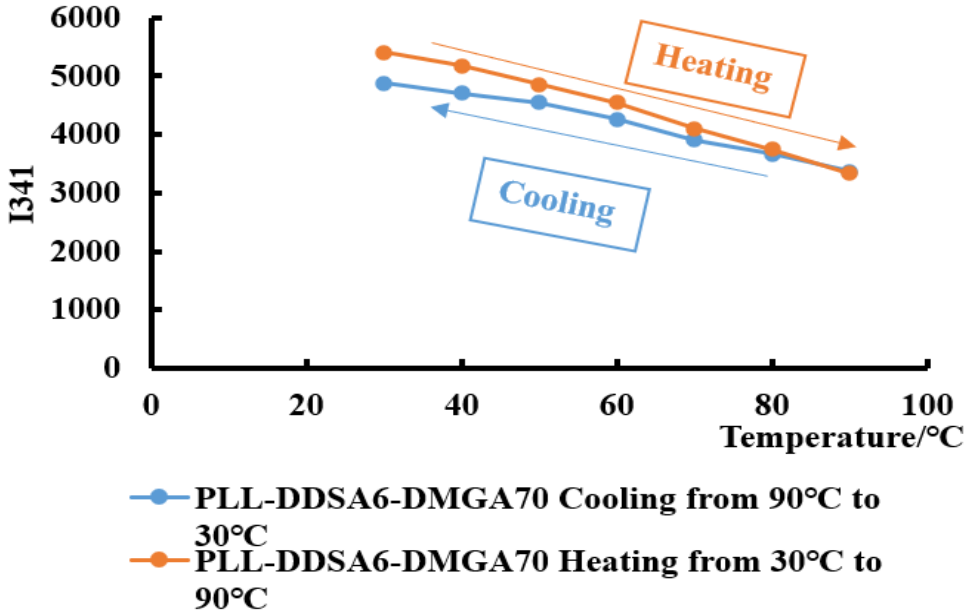


Figure 4.7 a b c d Polymer mixed with protein and cycling heating and cooling experiments from 30 ° C to 90 ° C

According to the results of the diagram, it can be observed that after the addition of the polymer, even after several cycles of heating and cooling experiments at high temperatures (90 ° C), the protein can still maintain its S1 conformation, and successfully refold at lower temperatures, returning to its natural state. This phenomenon further confirmed that the mechanism by which polymers inhibit protein aggregation is to maintain the protein in reversible conformation (S1) and effectively prevent its transition to irreversible conformation (S2). The mechanism diagram of the experiment is shown in Figure 4.8 (a) and (b).

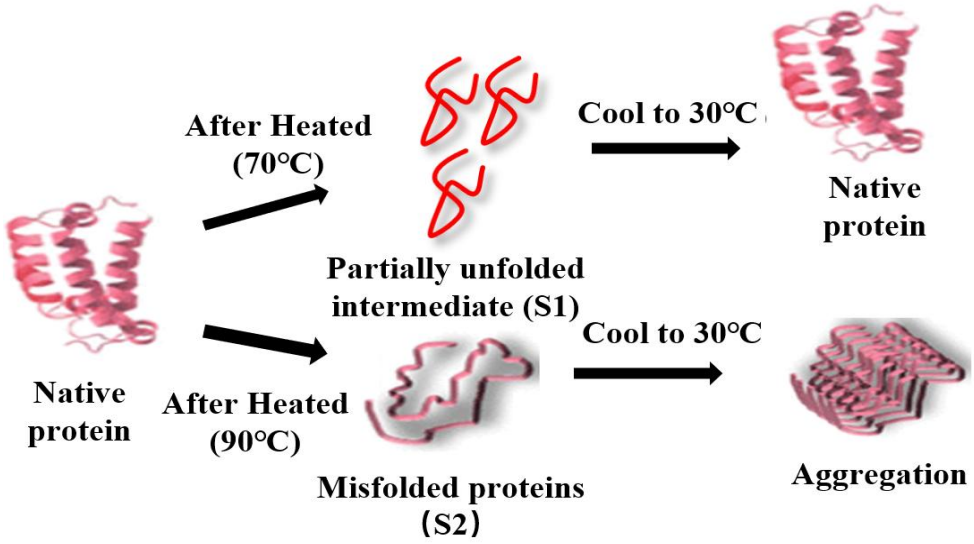


Figure 4.8 a Diagram of aggregation mechanism of proteins at different temperatures without the addition of polymers

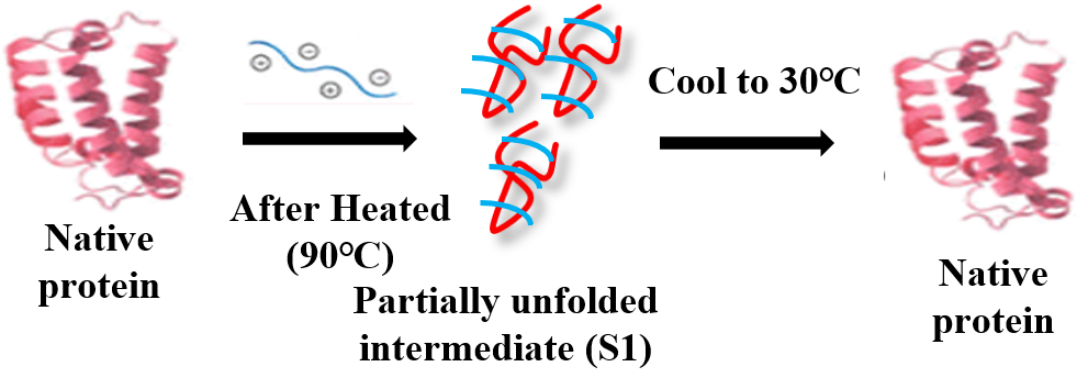


Figure 4.8 b Diagram of aggregation mechanism of proteins at 90 ℃ with the addition of polymers

4.3 Conclusion

In this part of the experiment, I designed two experiments to further explore the ways for polymer inhibition of protein aggregation.

Previous studies have confirmed that the weak interaction between polymers and proteins is one of the main reasons why polymers can inhibit protein aggregation. [73] When the weak interaction is slightly enhanced, such as introducing hydrophobic monomers, the efficiency of protecting protein aggregation can be further improved.[73] And this is consistent with what I found in my previous experiments. In this part of the two experiments, the polymer is through the subtle weakly interacting with proteins to maintain the chemical structure of protein and related chemical conformation.

Firstly, the secondary structure of some proteins was determined in detail by nuclear magnetic resonance (NMR) spectroscopy. The results showed that after denaturation, the secondary structure of the protein was almost destroyed and turned into a random spiral coil, a change that is almost impossible to observe in the spectrum. However, when the polymer was introduced, the protein retained most of the secondary structure and part of the higher-order structure under heating conditions, effectively preventing protein aggregation. This leads to the first conclusion that the inhibition of protein aggregation by polymers essentially preserves the secondary structure of their proteins, as well as part of the higher-order structure.

In the second experiment, first I detected the irreversible aggregation temperature of lysozyme by using phase diagram technology, and the experimental results were highly consistent with the theoretical prediction. In addition, two different conformations (S1) and (S2) of the protein were found in the experiment. Then, I further explained the reason why the polymer can inhibit protein aggregation by performing cyclic heating and cooling experiments, utilizing the two conformations (S1) and (S2) in the phase diagram. This is achieved by maintaining the reversible conformation of the protein and preventing its transition to an irreversible conformation.

Chapter 5

Conclusion

In chapter 2, I synthesized a polyampholytes polymer (PLL-SA and PLL-DDSA-SA) and investigated its inhibitory effect on protein aggregation. First, I synthesized polyampholytes electrolyte (PLL-SA) and enhanced its hydrophobicity by gradually increasing the proportion of DDSA (2%, 5%, 8% and 10%, respectively) to form a series of PLL-DDSA-SA polymers (in which the SA content was fixed at 65%). Subsequently, I characterized these polymers by 1H-NMR, and the results confirmed that the required amounts of SA and DDSA were successfully embedded into the PLL structure.

To evaluate the ability of these polymers to inhibit protein aggregation, I employed dynamic light scattering (DLS) techniques. The results show that lysozyme forms large aggregates when heated alone, and the pre-addition of polymers can completely inhibit this aggregation. In further experiments, I examined the residual enzyme activity of Micrococcus lysosomes before and after heating and after adding the polymer. The results showed that the presence of polymer significantly retained enzyme activity, and with the increase of DDSA content, the enzyme activity also showed a significant increase. PLL-DDSA8-SA65 polymers retain more than 80% of the enzyme activity, fully demonstrating the great potential of these polymers. In addition, further studies have explored the protective effect of these polymers against other proteins (lactate dehydrogenase), and the results likewise show that the polymers can provide good protection even at low concentrations.

In summary, I have successfully prepared a series of polyampholytes electrolytes (PLL-DDSA-SA) with high protein protection efficiency. These polymers can still maintain more than 80% enzyme activity under high temperature heating conditions, and the introduction of hydrophobicity significantly improves their protective effect. These findings show that such polymers have significant advantages in protecting proteins from extreme environmental impacts and are expected to be widely used in protein

biopharmaceuticals and drug delivery systems.

In chapter 3, first, I successfully synthesized DMGA and confirmed its high similarity to SA through detailed chemical structure characterization. On this basis, I systematically observed and recorded the dynamic changes of protein aggregation state by reacting DMGA with target proteins under a series of carefully designed experimental conditions. To ensure the accuracy and reliability of the experimental results, I specially set SA as the control group. Through comparative analysis of the data, the remarkable effect of this kind of charged polymer on inhibiting protein aggregation was verified. Subsequently, I systematically tested the inhibition efficiency of this polymer at different concentrations, and the results showed that this polymer can maintain excellent inhibition effects even at very low concentrations.

Further, I increased the proportion of acid anhydride in the polymer, and the experimental results showed that the polymer with high acid anhydride content was more effective in inhibiting protein aggregation. This phenomenon may be attributed to an increase in the surface charge density of the polymer, which enhances the electrostatic interaction between the protein and the polymer, ultimately inhibiting the aggregation of the protein. Finally, I optimized the ratio of anhydrides to hydrophobic groups, further improving the efficiency of this polymer in inhibiting protein aggregation.

In chapter 4, I designed two experiments to further explore the ways for polymer inhibition of protein aggregation.

Previous studies have confirmed that the weak interaction between polymers and proteins is one of the main reasons why polymers can inhibit protein aggregation.[121] When the weak interaction is slightly enhanced, such as introducing hydrophobic monomers, the efficiency of protecting protein aggregation can be further improved.[121] And this is consistent with what I found in my previous experiments. In this part of the two experiments, the polymer is through the subtle weakly interacting with proteins to maintain the chemical structure of protein and related chemical conformation.

Firstly, the secondary structure of some proteins was determined in detail by nuclear magnetic resonance (NMR) spectroscopy. The results showed that

after denaturation, the secondary structure of the protein was almost destroyed and turned into a random spiral coil, a change that is almost impossible to observe in the spectrum. However, when the polymer was introduced, the protein retained most of the secondary structure and part of the higher-order structure under heating conditions, effectively preventing protein aggregation. This leads to the first conclusion that the inhibition of protein aggregation by polymers essentially preserves the secondary structure of their proteins, as well as part of the higher-order structure.

In the second experiment, first I detected the irreversible aggregation temperature of lysozyme by using phase diagram technology, and the experimental results were highly consistent with the theoretical prediction. In addition, two different conformations (S1) and (S2) of the protein were found in the experiment. Then, I further explained the reason why the polymer can inhibit protein aggregation by performing cyclic heating and cooling experiments, utilizing the two conformations (S1) and (S2) in the phase diagram. This is achieved by maintaining the reversible conformation of the protein and preventing its transition to an irreversible conformation.

Reference

1. Sofronova, A. A., Izumrudov, V. A., Muronetz, V. I., & Semenyuk, P. I. (2017). Similarly charged polyelectrolyte can be the most efficient suppressor of the protein aggregation. *Polymer*, *108*, 281-287.
2. Roque, A. C. A., Lowe, C. R., & Taipa, M. Â. (2004). Antibodies and genetically engineered related molecules: production and purification. *Biotechnology progress*, *20*(3), 639-654.
3. Global Report on Diabetes, World Health Organization,Geneva 27, Switzerland 2016.
4. Li, T., Cao, H. X., & Ke, D. (2021). Type 2 Diabetes Mellitus Easily Develops into Alzheimer's Disease via Hyperglycemia and Insulin Resistance. *Current Medical Science*, 1-7.
5. Okamura, S., Hayashino, Y., Kore-Eda, S., & Tsujii, S. (2013). Localized amyloidosis at the site of repeated insulin injection in a patient with type 2 diabetes. *Diabetes Care*, *36*(12), e200-e200.
6. Moore, J. M., Patapoff, T. W., & Cromwell, M. E. (1999). Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody to vascular endothelial growth factor. *Biochemistry*, *38*(42), 13960-13967.
7. Answering Common Questions About Insulin For T1D
8. Herceptin (trastuzumab) Cancer Medication - Cancer Health
9. L.V. Kalia, S.K. Kalia, P.J. McLean, A.M. Lozano, A.E. Lang, a-Synuclein oligomers and clinical implications for Parkinson disease, Ann. Neurol. 73 (2013) 155e169, http://dx.doi.org/10.1002/ana.23746.
10. K.H. Ashe, A. Aguzzi, Prions, prionoids and pathogenic proteins in Alzheimer disease, Prion 7 (2013) 55e59, http://dx.doi.org/10.4161/pri.23061.
11. Dai, X., Zhao, D., Matsumura, K., & Rajan, R. (2023). Polyampholytes and Their Hydrophobic Derivatives as Excipients for Suppressing Protein Aggregation. ACS Applied Bio Materials, 6(7), 2738-2746.
12. Alam, P., Siddiqi, K., Chturvedi, S. K., & Khan, R. H. (2017). Protein aggregation: from background to inhibition strategies. *International journal of biological macromolecules*, *103*, 208-219.
13. Bross, P., Corydon, T. J., Andresen, B. S., Jørgensen, M. M., Bolund, L., & Gregersen, N. (1999). Protein misfolding and degradation in genetic diseases. *Human mutation*, *14*(3), 186-198.
14. Di Domenico, F., & Lanzillotta, C. (2022). The disturbance of protein synthesis/degradation homeostasis is a common trait of age-related neurodegenerative disorders. *Advances in Protein Chemistry and Structural Biology*, *132*, 49-87.
15. Díaz-Villanueva, J. F., Díaz-Molina, R., & García-González, V. (2015). Protein folding and mechanisms of proteostasis. *International journal of molecular*

sciences, *16*(8), 17193-17230.
16. Hipp, M. S., Park, S. H., & Hartl, F. U. (2014). Proteostasis impairment in protein-misfolding and-aggregation diseases. *Trends in cell biology*, *24*(9), 506-514.
17. Siddiqi, M. K., Alam, P., Chaturvedi, S. K., Shahein, Y. E., & Khan, R. H. (2017). Mechanisms of protein aggregation and inhibition. *Front Biosci (Elite Ed)*, *9*(1), 1-20.
18. Hatami, A., Monjazeb, S., Milton, S., & Glabe, C. G. (2017). Familial Alzheimer's disease mutations within the amyloid precursor protein alter the aggregation and conformation of the amyloid-β peptide. *Journal of Biological Chemistry*, *292*(8), 3172-3185.
19. Currais, A., Fischer, W., Maher, P., & Schubert, D. (2017). Intraneuronal protein aggregation as a trigger for inflammation and neurodegeneration in the aging brain. *The FASEB Journal*, *31*(1), 5-10.
20. Alam, P., Siddiqi, K., Chturvedi, S. K., & Khan, R. H. (2017). Protein aggregation: from background to inhibition strategies. *International journal of biological macromolecules*, *103*, 208-219.
21. Chiti, F., & Dobson, C. M. (2006). Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.*, *75*, 333-366.
22. Ross, C. A., & Poirier, M. A. (2004). Protein aggregation and neurodegenerative disease. *Nature medicine*, *10*(7), S10-S17.
23. Chi, E. Y., Krishnan, S., Randolph, T. W., & Carpenter, J. F. (2003). Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharmaceutical research, 20(9), 1325-1336.
24. Wiggins, P. M. (1997). Hydrophobic hydration, hydrophobic forces and protein folding. Physica A: Statistical Mechanics and its Applications, 238(1-4), 113-128.
25. Damodaran, S. (2008). Amino acids, peptides and proteins. Fennema's food chemistry, 4, 425-439.
26. Dasgupta, S., & Bell, J. A. (1993). Design of helix ends: Amino acid preferences, hydrogen bonding and electrostatic interactions. International Journal of Peptide and Protein Research, 41(5), 499-511.
27. Rana, A., Samtiya, M., Dhewa, T., Mishra, V., & Aluko, R. E. (2022). Health benefits of polyphenols: A concise review. Journal of Food Biochemistry, 46(10), e14264.
28. Belščak-Cvitanović, A., Durgo, K., Huđek, A., Bačun-Družina, V., & Komes, D. (2018). Overview of polyphenols and their properties. In Polyphenols: Properties, recovery, and applications (pp. 3-44). Woodhead Publishing.
29. Ngoungoure, V. L. N., Schluesener, J., Moundipa, P. F., & Schluesener, H. (2015). Natural polyphenols binding to amyloid: A broad class of compounds to treat different human amyloid diseases. Molecular nutrition & food research, 59(1), 8-20.
30. Ahmad, B., Borana, M. S., & Chaudhary, A. P. (2017). Understanding curcumin-

induced modulation of protein aggregation. International journal of biological macromolecules, 100, 89-96.
31. Tabasi, M., Maghami, P., Amiri-Tehranizadeh, Z., Saberi, M. R., & Chamani, J. (2023). New perspective of the ternary complex of nano-curcumin with β-lactoglobulin in the presence of α-lactalbumin: Spectroscopic and molecular dynamic investigations. Journal of Molecular Liquids, 392, 123472.
32. Ono, K., Hasegawa, K., Naiki, H., & Yamada, M. (2004). Curcumin has potent anti-amyloidogenic effects for Alzheimer's β-amyloid fibrils in vitro. Journal of neuroscience research, 75(6), 742-750.
33. Wang, S. S. S., Liu, K. N., & Lee, W. H. (2009). Effect of curcumin on the amyloid fibrillogenesis of hen egg-white lysozyme. Biophysical chemistry, 144(1-2), 78-87.
34. Chen, F. P., Li, B. S., & Tang, C. H. (2015). Nanocomplexation between curcumin and soy protein isolate: Influence on curcumin stability/bioaccessibility and in vitro protein digestibility. Journal of Agricultural and Food Chemistry, 63(13), 3559-3569.
35. Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. Nature, 475(7356), 324-332.
36. Kim, Y. E., Hipp, M. S., Bracher, A., Hayer-Hartl, M., & Ulrich Hartl, F. (2013). Molecular chaperone functions in protein folding and proteostasis. Annual review of biochemistry, 82(1), 323-355.
37. Bardwell, J. C., & Jakob, U. (2012). Conditional disorder in chaperone action. Trends in biochemical sciences, 37(12), 517-525.
38. Chaudhuri, T. K., & Paul, S. (2006). Protein-misfolding diseases and chaperone-based therapeutic approaches. The FEBS journal, 273(7), 1331-1349.
39. Niforou, K., Cheimonidou, C., & Trougakos, I. P. (2014). Molecular chaperones and proteostasis regulation during redox imbalance. Redox biology, 2, 323-332.
40. Tyedmers, J., Mogk, A., & Bukau, B. (2010). Cellular strategies for controlling protein aggregation. Nature reviews Molecular cell biology, 11(11), 777-788.
41. Morano, K. A., Grant, C. M., & Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in Saccharomyces cerevisiae. Genetics, 190(4), 1157-1195.
42. Morgan, P. E., Treweek, T. M., Lindner, R. A., Price, W. E., & Carver, J. A. (2005). Casein proteins as molecular chaperones. Journal of agricultural and food chemistry,

53(7), 2670-2683.
43. Qu, K., Yuan, Z., Wang, Y., Song, Z., Gong, X., Zhao, Y., ... & Wang, L. (2022). Structures, properties, and applications of zwitterionic polymers. ChemPhysMater, 1(4), 294-309.
44. Qian, H., Wang, K., Lv, M., Zhao, C., Wang, H., Wen, S., ... & Zhong, Y. (2022). Recent advances on next generation of polyzwitterion-based nano-vectors for targeted drug delivery. Journal of Controlled Release, 343, 492-505.
45. Li, Q., Wen, C., Yang, J., Zhou, X., Zhu, Y., Zheng, J., ... & Zhang, P. (2022). Zwitterionic biomaterials. Chemical reviews, 122(23), 17073-17154.
46. Zhang chong, & Lv Hua. (2018). Synthesis and application of protein-poly (amino acid) conjugates. ACTA POLYMERICA SINICA.
47. Schellekens, H., Hennink, W. E., & Brinks, V. (2013). The immunogenicity of polyethylene glycol: facts and fiction. Pharmaceutical research, 30(7), 1729-1734.
48. Rajan, R., & Matsumura, K. (2015). A zwitterionic polymer as a novel inhibitor of protein aggregation. Journal of Materials Chemistry B, 3(28), 5683-5689.
49. Rajan, R., & Matsumura, K. (2017). Inhibition of protein aggregation by zwitterionic polymer-based core-shell nanogels. Scientific reports, 7(1), 1-9.
50. . Kudaibergenov, S. E. (1999). Recent advances in the study of synthetic polyampholytes in solutions. Polymer Latexes-Epoxide Resins-Polyampholytes, 115-197.
51. Bekturov, E. A., Kudaibergenov, S. E., & Rafikov, S. R. (1990). Synthetic polymeric ampholytes in solution. Journal of Macromolecular Science—Reviews in Macromolecular Chemistry and Physics, 30(2), 233-303.
52. Laschewsky, A. (2014). Structures and synthesis of zwitterionic polymers. Polymers, 6(5), 1544-1601.
53. Hollecker, M., & Creighton, T. E. (1982). Effect on protein stability of reversing the charge on amino groups. Biochimica et Biophysica Acta (Bba)-Protein Structure and Molecular Enzymology, 701(3), 395-404.
54. Xu, Y., Chen, K., Gao, C., Wang, J., Wu, W., Zhao, F., ... & Zhao, Y. (2022). High water-retaining, antifreeze micro-supercapacitor without encapsulation for all-weather. Journal of Power Sources, 545, 231909.
55. Kudaibergenov, S. E., Nuraje, N., & Khutoryanskiy, V. V. (2012). Amphoteric nano-, micro-, and macrogels, membranes, and thin films. Soft Matter, 8(36), 9302-9321.
56. Dubey, S., Mody, N., Sharma, R., Agrawal, U., & Vyas, S. P. (2016). Nanobiomaterials: Novel nanoplatforms for protein and peptide delivery. In Nanobiomaterials in drug delivery (pp. 111-146). William Andrew Publishing.
57. Bernards, M., & He, Y. (2014). Polyampholyte polymers as a versatile zwitterionic biomaterial platform. Journal of Biomaterials Science, Polymer Edition, 25(14-15), 1479-1488.
58. Saimura, M., Takehara, M., Mizukami, S., Kataoka, K., & Hirohara, H. (2008).

Biosynthesis of nearly monodispersed poly (ε-l-lysine) in Streptomyces species. Biotechnology letters, 30(3), 377-385.
59. Hiraki, J., Ichikawa, T., Ninomiya, S. I., Seki, H., Uohama, K., Seki, H., ... & Barnett Jr, J. W. (2003). Use of ADME studies to confirm the safety of ε-polylysine as a preservative in food. Regulatory Toxicology and Pharmacology, 37(2), 328-340.
60. USFDA, Agency Response Letter GRAS Notice No. GRN 000135. In GRAS Notice No. GRN 000135, U.S. Food and Drug Administration: Washington, D.C., 2004.
61. Yu, H., Huang, Y., & Huang, Q. (2010). Synthesis and characterization of novel antimicrobial emulsifiers from ε-polylysine. Journal of agricultural and food chemistry, 58(2), 1290-1295.
62. Rajan, R., Suzuki, Y., & Matsumura, K. (2018). Zwitterionic polymer design that inhibits aggregation and facilitates insulin refolding: mechanistic insights and importance of hydrophobicity. Macromolecular bioscience, 18(6), 1800016.
63. Mora-Huertas, C. E., Fessi, H., & Elaissari, A. (2010). Polymer-based nanocapsules for drug delivery. International journal of pharmaceutics, 385(1-2), 113-142.
64. Shimizu, S., & Matubayasi, N. (2014). Hydrotropy: Monomer–Micelle equilibrium and minimum hydrotrope concentration. The Journal of Physical Chemistry B, 118(35), 10515-10524.
65. Zhao, H., Lin, Z. Y., Yildirimer, L., Dhinakar, A., Zhao, X., & Wu, J. (2016). Polymer-based nanoparticles for protein delivery: design, strategies and applications. Journal of Materials Chemistry B, 4(23), 4060-4071.
66. Aliyan, A., Cook, N. P., & Martí, A. A. (2019). Interrogating amyloid aggregates using fluorescent probes. Chemical reviews, 119(23), 11819-11856.
67. Lee, C. H. (2010). 1-anilinonaphthalene-8-sulfonate (ANS); a versatile fluorescent probe from protein folding study to drug design. BioWave, 12(6), 1.
68. Cavanagh, J.; Fairbrother, W. J.; Palmer, A. G., III; Rance, M.; Skelton, N. J. Protein NMR Spectroscopy, 2nd ed.; Academic Press: Burlington, 2007.There is no corresponding record for this reference
69. Boyd, J.; Dobson, C. M.; Redfield, C. Assignment of Resonances in the 1H NMR Spectrum of Human Lysozyme. Eur. J. Biochem. 1985, 153, 383– 396, DOI: 10.1111/j.1432-1033.1985.tb09314.x
70. Cheetham, J. C.; Artymiuk, P. J.; Phillips, D. C. Refinement of an Enzyme Complex with Inhibitor Bound at Partial Occupancy: Hen Egg-White Lysozyme and Tri-N-Acetylchitotriose at 1.75 Åresolution. J. Mol. Biol. 1992, 224, 613– 628, DOI: 10.1016/0022-2836(92)90548-X
71. Wang, Y.; Bjorndahl, T. C.; Wishart, D. S. Letter to the Editor: Complete 1H and

Non-Carbonylic 13C Assignments of Native Hen Egg-White Lysozyme. J. Biomol. NMR 2000, 17, 83– 84, DOI: 10.1023/A:1008359624072

72. Wang, F., Sheng, S., Ge, X., Huang, H., & Ma, Q. (2019). Exploring the interaction between milrinone drug and lysozyme by fluorescence spectroscopy and differential scanning calorimetry. Journal of Thermal Analysis and Calorimetry, 135, 2849-2856.
73. Rajan, R., Furuta, T., Zhao, D., & Matsumura, K. (2024). Molecular mechanism of protein aggregation inhibition with sulfobetaine polymers and their hydrophobic derivatives. Cell Reports Physical Science.
74. Kuznetsova IM, Turoverov KK, Uversky VN. Use of the phase diagram method to analyze the protein unfolding-refolding reactions: fishing out the "invisible" intermediates. Journal of proteome research. 2004 Jun 14;3(3):485-94.

List of achievements

Journals

Dai, X., Zhao, D., Matsumura, K., & Rajan, R. (2023). Polyampholytes and Their Hydrophobic Derivatives as Excipients for Suppressing Protein Aggregation. *ACS Applied Bio Materials*, *6*(7), 2738-2746.

Rajan, R., Kumar, N., Zhao, D., Dai, X., Kawamoto, K., & Matsumura, K. (2023). Polyampholyte-Based Polymer Hydrogels for the Long-Term Storage, Protection and Delivery of Therapeutic Proteins. Advanced Healthcare Materials, 12(17), 2203253.

Conferences

Poster "Design the polyampholytes in inhibiting protein aggregation and exploring its mechanism" Xianda Dai, Kazuaki Matsumura. The 34th Annual Meeting of MRS-Japan. December,16, 2024

Acknowledgement

During my PhD study at JAIST, first I would like to express my heartfelt thanks to my supervisor, Professor Kazuaki Matsumura, for letting me become a member of Matsumura Lab. In addition, I would like to thank the professor for his valuable advice and patient guidance during my doctoral study. It was under the guidance of the professor that I was able to continuously improve myself and finally complete my doctoral study.

Next, I would like to thank my assistant professor, Dr. Robin Rajan. During the doctoral study, he gave me a lot of valuable advice and selfless help from a professional perspective. I am very grateful to him, with his help, some of my scientific research problems have been solved. I am honored to have known such an excellent teacher during my doctoral studies.

Next, I would like to thank the teachers of my doctoral pre-defense, Prof. Kurisawa, Prof. Miyako, Prof. Matsumi, and Prof. Nakaji. I would like to thank them for giving me some professional criticism and suggestions during my doctoral defense, and thank them for their patient guidance, which made my research more perfect and professional.

In addition, sincere thanks to all the laboratory members of Matsumura Laboratory. At the beginning, I was not familiar with the new environment. Thank them for their selfless help and support when I encountered difficulties, so that I felt the warmth of home in a foreign country. Also, I would like to thank my Chinese friends at JAIST who selflessly helped me when I was in trouble. It was their company that made me feel less alone.

Finally, I would like to thank my family, who have given me the greatest encouragement and support. Especially my parents, without whom I could not have come to Japan to complete my PhD. They are my spiritual support in a foreign land, and I am very grateful to them for believing in me. They always teach me to be an excellent person with knowledge and ability as well as character.

Xianda DAI

January, 2025