# 行政院國家科學委員會專題研究計畫　期中進度報告

## 蛋白質摺疊與聚集(1/3)

計畫類別：個別型計畫
計畫編號：NSC94-2112-M-009-039-
執行期間：94 年 08 月 01 日至 95 年 07 月 31 日
執行單位：國立交通大學生物科技學系(所)

計畫主持人：張家靖

報告類型：精簡報告
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中　華　民　國 95 年 5 月 26 日

# 行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告 ∨期中進度報告

## 蛋白質摺疊與聚集 (1/3)

計畫類別：∨ 個別型計畫　　□ 整合型計畫
計畫編號：NSC 94－2112－M－009－039
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計畫主持人：張家靖
共同主持人：
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：∨精簡報告　□完整報告

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執行單位：國立交通大學生物科技學系

中　　華　　民　　國 95 年 05 月 22 日

# 蛋白質摺疊與聚集（1/3）

Summary the achievement of this project.

Within this granted year, we have found that the reactions of protein spontaneous folding and diffusion-limited aggregation are antagonistic in nature. Therefore, we reported this phenomena and published in Biochemical and Biophysical Research Communication (2005) (reference 1). Meanwhile, we also found that this antagonistic reaction can be found not only in Lysozyme system but also in Growth hormone system. Namely, this reaction may be an universal behavior during the protein folding. We reported this finding to Journal of Cell biochemical and Biophysics and the manuscripts is under revision now (reference 2). We also wrote a review article to summarize the current real protein folding mechanism and this will be published in "Protein folding: new research" (2006) (reference 3).

In the coming granted years we will focus on the mechanism studies of the factors, such as solvent and/or intrinsic amino acids, that will affect the protein aggregation process.

Besides the fundamental studies of protein folding, we also found that we can change the properties of protein and turned into magnetic one. This has been report in Biochemical and Biophysical Research Communication (2006) (reference 4). Meanwhile, this protein revealed self-assembly property and formed protein rod in restricted nano-patterning silicon substrate. This has been accepted to publish in Applied Physics Letter (2006) (reference 5).


1. **Chang CC***, Lin PY, Yeh XC, Deng KH, Ho YP and Kan LS* (2005) Protein folding stabilizing time measurement: a direct folding process and three-dimensional random walk simulation. *Biochem. Biophys. Res. Commun.* 328, 845-850. (SCI) (NSC 93-2112-M-259-001)
2. **Chang CC**, Wu CY, Chen PH, Chu HL, Ho CC, Chou FS, Sun MC, Ho YP, Kan LS (2006) Recombinant growth hormone folding: an antagonistic reaction of spontaneous folding and diffusion limited aggregation. (in revision)
3. **Chang CC** and Kan L.-S (2006), Protein folding and aggregation. In *Protein folding: new research*, Nova publisher, New York. (Accepted) (NSC 94-2112-M-009-039).
4. **Chang C.-C***, Lee S.-F.; Sun K.-W., Ho C.-C.; Chen Y.-T.; Chang C.-H., Kan L.-S. (2006) Mn,Cd-metallothionein-2: A room temperature magnetic protein. *Biochem. Biophys. Res. Commun.* 340, 1134-1138. (SCI) (NSC 94-2112-M-009-039).
5. **Chang C.-C**, Sun K.-W., Kan L.-S. kuan CH (2006) Guided Three-dimensional Molecular Self-assembly on Silicon Substrates. Applied Physics Letter (accepted). (SCI).


The following is the detail of the manuscript of

**Recombinant growth hormone folding: an antagonistic reaction of spontaneous folding and diffusion limited aggregation.**

Abstract:

The molecules in a protein solution, like any other particles in a solution, undergo Brownian motion and collide with each other constantly. The collisions may lead to protein precipitation when the situation favors aggregation. Thus for proteins to fold successfully in solution, the process has to complete before any significant collisions take place. We estimated the folding time of recombinant growth hormone (rGH) by studying the correlation time of a direct folding process from autocorrelation function analysis of the mean collision time and aggregation/soluble ratio of protein. It gives an estimate of the time limit before the protein aggregates. It is also a measure of time during which the protein folds into a stable state.

We found the protein folding stabilizing time of rGH system to be 29.7 to 14.8 μs (<±4%) between 309 to 293 K via direct folding experimental studies, supported by a three-dimensional random walk simulation of diffusion limited aggregation model. Aggregation is suppressed when the protein is folded to a stable form. Meanwhile, the optimal reaction temperature is at 301 K. Spontaneous folding and diffusion-limited aggregation are antagonistic in nature. The resultant aggresome, as suggested by Raman and mass spectroscopy, may have been formed by cross linkages of disulfide bonds and hydrophobic interactions.

**Introduction:**

During the protein folding process, some critical structures of protein may be formed sequentially in nano- to micro-seconds (1). The sequential folding phenomenon may reveal a possible and important mechanism of the initiation stage of protein folding (2). However, the observation of conformational reshuffling (3) and the formation of disulfide bridges (4, 5) during subsequent folding processes indicate the possibility of conformational instability during the initial folding stage. Thus, how soon a protein can stabilize itself becomes an important issue in its folding process.

The conformational instability of protein leads to intermolecular aggregation during a protein folding process (6). Such instability can induce protein conformational changes and aggregations, which subsequently, leading to diseases (7). These aggresomes may be caused by hydrophobic interactions and/or intermolecular disulfide bonds formation. However, these aggregation processes do not occur in low concentration (8), and hence they may be regulated.

According to the first-order-like state transition model, proteins may undergo multiple folding paths from an unfolded state to a native state (3, 9~12). However, when the protein is folded in a non-continuous manner (i.e. reaction crosses state transition line) both unfolded state and molten globule state can be observed at the same time when the reaction reaches a thermal equilibrium (3). Conventional ultra fast folding studies (13~15) have focused their attention on the protein structural information rather than its stability, i.e., more from a static rather than a dynamic viewpoint. Therefore, small peptides (13,14) or single domain of proteins (15) were studied to avoid the complexity of real protein folding. However, it remained unclear whether a real protein was stable during the course of such experimental approaches. It is thus important to reveal the folding mechanisms via various proper folding paths and observe their stability and time courses.

As indicated by energy landscape model (16), protein may be trapped into local energy minima during the course of protein folding. However, most of the protein should culminate in the native state, the lowest energy state. Thus, for such a single particle system as in protein folding, the native state should be the most probable state to be observed. On the contrary, in a high concentration system, most of the protein will aggregate during the folding process *in vitro* (8). The size of the clustered aggregation is larger than those held together by regular, weaker forces, and thus may cause precipitation (10,11). The kinetic mechanism of the aggregation is likely to be a diffusion limited aggregation process (17, 18). Forrest and Witten have further described the process by a density correlation of power-law form (19). Previous experiments have indicated that there was no aggregation of unfolded protein observed in lower concentration (8). The limitation on aggregation may be described by an order parameter proposed to indicate the status of a protein folding (3). Namely, the protein may spontaneously fold or self-assemble into a stable state, during the diffusion process.

To verify this proposed mechanism, both experimental approaches and three-dimensional random walk simulations have been studied. Direct folding study of recombinant growth hormone (rGH) and autocorrelation analysis indicate that the spontaneous folding time is around 29.7 to 14.8 μs. The optimal folding temperature is 301 K. Molecular simulation results suggest that the protein folding

reaction is a competitive process between self-stabilization (spontaneously folding) and diffusion limited aggregation (DLA). The formation of aggresomes can be ascribed to mis-linkages of disulfide bonds and hydrophobic interactions, as observed from MALDI-TOF mass spectra and Raman spectra.

## Material and Methods

### Materials and buffers:

All chemicals were obtained from Merck Ltd. (Rahway, NJ). Denature/unfold buffer contains 4.5 M urea with 10 mM Tris base, 0.1 M dithiothreitol (DTT), 0.1 % mannitol and 0.5 mM protease inhibitor - Pefabloc. The native buffer is 10 mM Tris HCl at pH 8.8.

### Expression of recombinant fish growth hormone.

*E. coli* carrying recombinant yellow grouper growth hormone (rGH) expression plasmid was obtained from Dr. C. Y. Chang and was grown in LB broth until the optical absorption at $OD_{600}$ reached 0.3. Cells were then grown in LB media with the supplement of 0.1 mM IPTG at 37 °C for 16 h.

### Purification of inclusion body.

The protocol used for the isolation of inclusion body from *E. coli* and the unfolding was based on our previously described procedures (11). *E. coli* was harvested by centrifugation at 12000 g for 3 min. The cell pellet was resuspended and mixed well with ice-cold water and lysed with a cell disruptor (Constant System Ltd. Northants, UK). Total cell lysate was separated into soluble and insoluble portions by centrifugation at 12000 g for 3 min. The inclusion body was collected and washed twice with ice-cold double-distilled $H_2O$.

### Direct folding path.

The unfolded rGH (U), 41.4 mg/mL, was obtained by treating the rGH inclusion body with denaturing/unfolding buffer. This solution was left at room temperature for one hour and then was centrifuged at 12000 g for 3 min. Then direct dilution of unfolded rGH into 100 fold native buffer, mixed well at defined reaction temperature. The protein concentration in the soluble portion was determined by UV spectrometer at 277 nm with the extinction coefficient of rGH (11) and the dilution effect was calibrated with a serial dilution curve.

### Reduction/oxidation state of sulfur group of cysteine monitored by micro-Raman spectroscopy.

Raman experiments were carried out with the Ranishow 1000 micro-Raman system (Ranishow Plc, Gloucestershire, UK). Five μL rGH solutions of varying folding states (10 mg/mL) were lyophilized onto the surface of a platinum plate. The Raman spectra were collected around 2570 and 516 $cm^{-1}$. They represent the stretching frequencies of the S-H bond ($\nu_{S-H}$) of the reduced cysteine and the S-S bond ($\nu_{S-S}$) of the oxidized cystine of rGH, respectively (2, 20). The wave numbers of the Raman spectra were calibrated with silicon oxide. Each spectrum was compared with the same folding state buffer to identify the signal of protein and solvent, respectively.

### MALDI-TOF mass spectrometry.

The samples were prepared by mixing equal volume of matrix solution of

α-cyano-4-hydroxycinnamic acid (CHCA) (20 mg/mL) (Aldrich) in pure methanol and an analyte (protein) solution. A volume of one μL of the solution was deposited on a stainless sample probe and allowed to air-dry. Mass spectra of positively charged ions were acquired on a Bruker Autoflex instrument (Bruker Daltonik, Germany) operated in the linear mode. A total of 500-2500 single-shot spectra were accumulated from each sample. The laser power was adjusted to 37-38%. The acceleration voltage was 20 KV. The Flex analysis software packages (Bruker Daltonik, Germany) were used for data processing.

**Molecular simulation.**

The protein particles were randomly distributed in a defined three-dimensional box with a periodical boundary in our simulations. The size of the simulation box is determined by the cubic root of particle numbers multiplied by the mean distance between each protein. In order to reduce the calculation time, we use a periodical boundary for the simulation space. The optimal particle number for molecular simulation is 216 and the mean distance between each protein can be determined from the initial concentration of protein that indicated previously. The particles are allowed to move randomly and the span of each step is fixed and the relative walking time can be converted from Equation 6 (Results and Discussions). The gyration radius of particle is shrunk from 2.47 fold to native radius by a function of temperature dependent-exponential decay. Due to the gyration radii shrink, the aggregation activation factor was set as the similar equation (eq. 3 on next page). The rest of the protein particles, not interacting with each other, are treated as the ones spontaneously fold. By comparing the starting number of particles and the number of particles that remained (the number of spontaneous folds), the protein aggregation fraction can be obtained.

**Results and discussion:**

**Protein mean-collision/stabilization time determination by initial concentration of unfolded protein.**

In order to measure protein stabilization time directly, we conducted experiments to fold the proteins by way of direct folding path (3). The concentrations of the protein, diluted by native buffer, can be transformed to the mean-distance ( $\overline{X}$ ) between the proteins. According to a model of the three dimensional random-walk, the mean distance ( $\overline{X}$ ) is equal to the root mean square distance ($X_{rms}$); and the mean collision time for the protein diffusing across this distance can be determined by following equations.

$$\overline{X} \approx X_{rms} = \left\langle X^2 \right\rangle^{\frac{1}{2}} = \left(6Dt\right)^{\frac{1}{2}} \qquad \text{(Eq. 1)}$$

where D denotes the diffusion constant of protein as defined in Eq. 2,

$$D = \frac{kT}{6\pi\eta R_H} \qquad \text{(Eq. 2)}$$

where η denotes the viscosity of the protein solution. In this study, η assumes the viscosity of water (1 cp) since it does not change much in diluted condition (3). $R_H$ denotes the effective hydration radius of the protein. According to our and other studies, the unfolded protein is about 2.47 times larger than its

native protein (3, 9~12); and the radius of protein may be reduced by a function of exponential decay (21). Therefore, in this paper, we set the value of hydration radius of rGH from 7.53 nm to 2.75 nm ($R_0$) and the function of $R_H$ as:

$R_H(t) = R_0(1+1.47e^{-ct})$ (Eq. 3)

where c is the hydration radius collapse factor and the optimal value is 0.01 as described in the simulation section.

Therefore, the mean collision time during protein diffusive process can be expressed as following:

$$1.47X_{rms}^2 \times e^{-ct} - 6D_0 t + X_{rms}^2 = 0 \quad \text{(Eq. 4)}$$

$$t = \frac{cX_{rms}^2 + 6D_0 ProductLog[\frac{1.47ce^{-\frac{cX_{rms}^2}{6D_0}} X_{rms}^2}{6D_0}]}{6cD_0} \quad \text{(Eq. 5) where ProductLog can be expanded as}$$

following,

$$ProductLog[\frac{1.47ce^{-\frac{cX_{rms}^2}{6D_0}} X_{rms}^2}{6D_0}] = \frac{0.245cX_{rms}^2}{D_0} - \frac{0.1c^2X_{rms}^4}{D_0^2} + \frac{0.0455c^3X_{rms}^6}{D_0^3} \text{.....} \quad \text{(Eq. 6)}$$

Therefore, the mean collision/diffusion time for a specified initial concentration of unfolded protein can be determined and their values are 10.23 μs for 0.414 mg/ml and 119.12 μs for 10.35 μg/ml at 301 K, as an example. The mean collision time is also an upper limit for protein folding in the diffusive processes. Namely, if proteins do not fold and settle into a stable state during this period, they may aggregate and precipitate consequently.

**Both aggregated and soluble protein can be observed in direct folding path.**

RGH of varying concentration were unfolded in the unfolding buffers (10, 11) and then mixed rapidly with 100-fold native buffer (3, 10) by ultrasonication. Re-folding was then followed.

In order to test the temperature effect of direct folding reaction, the reaction mixtures were incubated at 293, 297,301, 305 and 309 K environments for 1 hour to reach equilibrium. This equilibrium time is $10^6$ to $10^9$ folds longer than the conventional protein folding studies (22~24). Therefore, the aggregation reaction has reached its equilibrium state. When the direct folding reaction reached equilibrium, the protein concentration in the soluble portion was determined by UV spectrometer. The aggregation fraction is equal to [1- (soluble protein/total protein)]. As described previously, the mean collision time for the rGH folding in 0.414 mg/ml was estimated to be 10.23 μs at 309 K. However, from the result shown in Figure 1a, the fraction of aggregation was found to be 0.70 at a mean collision time of 10.23 μs. It means that at this time, most of the protein was still in solution and not folded. However, when the initial concentration is decrease to 10.35 μg/ml the mean collision time was estimated to be 119.12 μs at 301 K. This time period is long enough for rGH to fold into stable state as the fractions of aggregation is decreased to 0.02 (Figure 1a). This 2% precipitation should be considered as system back ground for the protein molecules mixed well into the solution.

Meanwhile, in macroscopic experiment, it is impossible to synchronize all the particles of the proteins to fold from unfolded state to native state at the same time. Therefore, direct measurement from half fraction of protein aggregation is not rational. However, the aggregation reaction time relationship can be revealed by the autocorrelation function (ACF) of the protein aggregation fraction. Therefore, the function can be expressed as follows:

$\Gamma(\Delta t)=<C(t) \bullet C(t-\Delta t)>$ (Eq. 7)

where C(t) denotes the aggregation fraction at time t. The $\Delta t$ denotes the time interval between the observations. According to equation (7), the value of $\Gamma(\tau_c)$ is the half-width of the ACF (25) (Figure 1b). The $\tau_c$, the aggregation correlation time, is the time required for rGH to stabilize itself in a folding process. It is around 29.7 to 14.8 μs at 297K and 301 K, respectively, no matter whether the experimental reaction was synchronized or not. Thus in low concentrations, proteins have a chance to fold into a stable form before any collisions to occur. This may provide a reasonable interpretation at a molecular level for previously observed studies (8). Meanwhile these correlations among precipitation fractions versus lag time ($\Delta t$) can fit with logistic function (Figure 1b) very well. This function is a modified power law and this is consistent with Forrest and Witten's description (19). Namely, the aggregation of protein in the direct folding path protein obeys diffusion limited aggregation (DLA) model. However, during the folding reaction the thermal fluctuation may delay the folding process. There will be an optimization folding temperature, 301K of the direct folding process (Figure 2). Therefore, a DLA process is competing with the spontaneous folding process in a direct folding reaction in certain temperature. The detailed reaction mechanism will be elaborated in the simulation section below.

By comparison with the model of first-order-like state transition, the forbidden region of direct folding reaction may have been caused by the interactions among unfolded proteins. An over critical reaction regulated by solvent environment and denaturant may play a role to avoid effective collision between proteins and protein folds spontaneously during this period (10). These may explain the possible mechanism and function of molecular chaperons (3, 9~12) that help with protein folding.

**Three dimensional random walk simulation revealed that spontaneous folding and aggregation are antagonistic in nature.**

In order to reveal the molecular interaction during protein folding process, a three dimensional random walk simulation is used in this study. This model is quite different from Eden's model (19), the distribution of protein particles was considered as randomly distributed in a defined three-dimensional box with a periodical boundary. The size of the simulation box is determined by the particle numbers of simulation and the mean-distance $\overline{X}$ of Equation 1. The protein particles are considered to be in constant Brownian motion as described in Equations 1 and 2. Therefore, the event of protein collision can be monitored and be analyzed. In order to simulate the protein spontaneous folding process during the diffusion time an activation factor of protein aggregation was introduced as follows:

$$A(t)=\frac{e^{\frac{-t}{\tau}}}{(1+\log(T-273))} \quad \text{(Eq. 8)}$$

where τ denotes the folding time constant and the value is 500; T denotes the reaction temperature in Kelvin. We set the threshold to be 0.1. If the value *A* is lower than the threshold, proteins will not interact with each other.

From the simulation, we found that the aggregation fractions were similar for different numbers of particles (n). The optimal number of particles for simulation is about 216 for a reasonable amount of calculation time. We also find that those simulation results can fit well with our experimental results (Figure 1a). These suggested that the driving force for the unfolded proteins to move in the solution is thermal fluctuation and they move randomly. Meanwhile the particle size of protein (the hydration radius) shrinks during the diffusive process as indicated in Equation 3. By comparing the functions of activation factor (Equation 8) and mean collision time (Equation 6), we can find that the aggregation activation factor decreases faster than collision time when the reaction temperature is raised. However, the V shape of the trend of the correlation time ($\tau_c$) versus the reaction temperature (Figure 2) indicated that the reaction rate of protein spontaneously folding is not significantly faster than DLA. Therefore, we may conclude that the direct folding process of protein is a competitive process between a spontaneous folding and diffusion-limited aggregation (DLA).

**Aggresome caused by intermolecular disulfide bonds and hydrophobic interactions *in vitro*.**

In order to reveal the protein aggregation mechanism, both Raman and MALDI-TOF mass spectroscopy have been used. Raman spectra indicated that an aggregated rGH contained both oxidize disulfide bonds and reduced SH bonds (Figure 3). However, the soluble rGH obtained in the same reaction contained oxidized disulfide bonds only (Figure 3). These results implied that the intermolecular mis-linked disulfide bonds might exist in the aggresome. By sonicating these aggresome with an amphiphilic solvent acetonitrile, part of these aggregates can be dissolved. This indicates hydrophobic interaction plays important role in aggresome formations. The acetonitrile-soluble proteins can be analyzed with MALDI-TOF mass spectrometer, indicating that stable monomer, dimer, trimer and tetramer of rGH could be observed (Figure 4) in the precipitated portion, but not in the soluble portion (data not shown). Therefore, these aggregates of rGH are caused by disulfide mis-linkages and hydrophobic interactions *in vitro.* However, a native aggresome, such as inclusion body of *E. coli*, did not show to contain oligomer of proteins by using the same procedures (data not shown). Comparing both *in vitro* and *in vivo* experiments we conclude that the hydrophobic interaction is the majority factor for aggresome formation. Therefore, suitable protection chaperons and enzymes preventing mis-linked disulfide bonds formation are necessary *in vivo*.

In summary, this is the first study by which the stabilization time of protein folding has been observed directly and quantified. It is around 29.7 to 14.8 μs, depending on the reaction temperature. The protein folding mechanism in a cell free system is an antagonistic reaction between spontaneous folding and DLA. The aggregation of protein during folding process may be caused by disulfide bonds mis-linkage and hydrophobic interactions.

**Acknowledgements**

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**Figure legends:**

Figure 1. Fraction of protein aggregation versus diffusion collision time at various temperatures. (a) T =301 K. The solid squares and dotted line denoted the experimental results and the simulation results, respectively. (b) The auto-correlation function of the fraction of protein versus diffusion collision time. The horizontal and vertical lines in the Figure denoted the fitting of the auto-correlation function by logistic function. The $\tau_c$ represented the correlation time for which $\Gamma(\tau_c)=1/2\ \Gamma(0)$.

Figure 2. The temperature dependent protein folding time.

Figure 3. Raman shift of rGH aggregates and soluble form. (a) The S-S ($\nu_{S-S}$) stretching region. (b) The S-H ($\nu_{S-H}$) stretching region. The gray line and black line denoted precipitated and soluble rGH, respectively.

Figure 4. MALDI-TOF mass spectra of acetonitrile soluble rGH aggregates. The molecular weight of rGH. is 22.32 KD. There are monomer, dimmer, trimer and tetramer of the rGH. Where the m denoted the molecule of rGH.

Figure 1a.

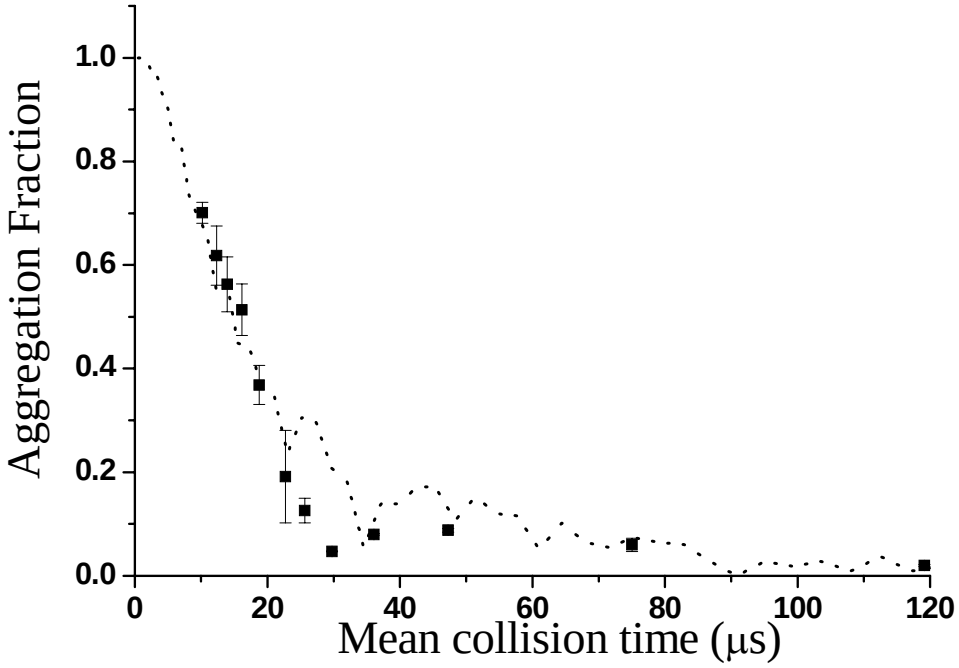



Figure1b.

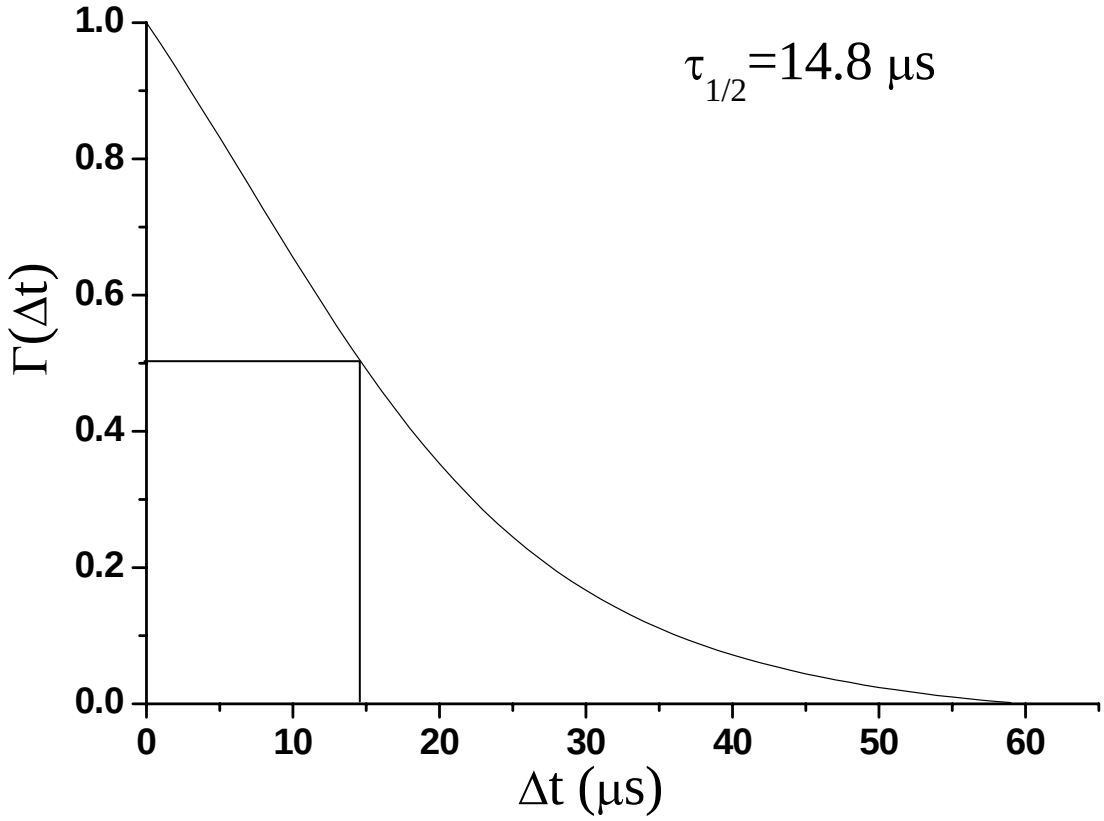


Figure 2.

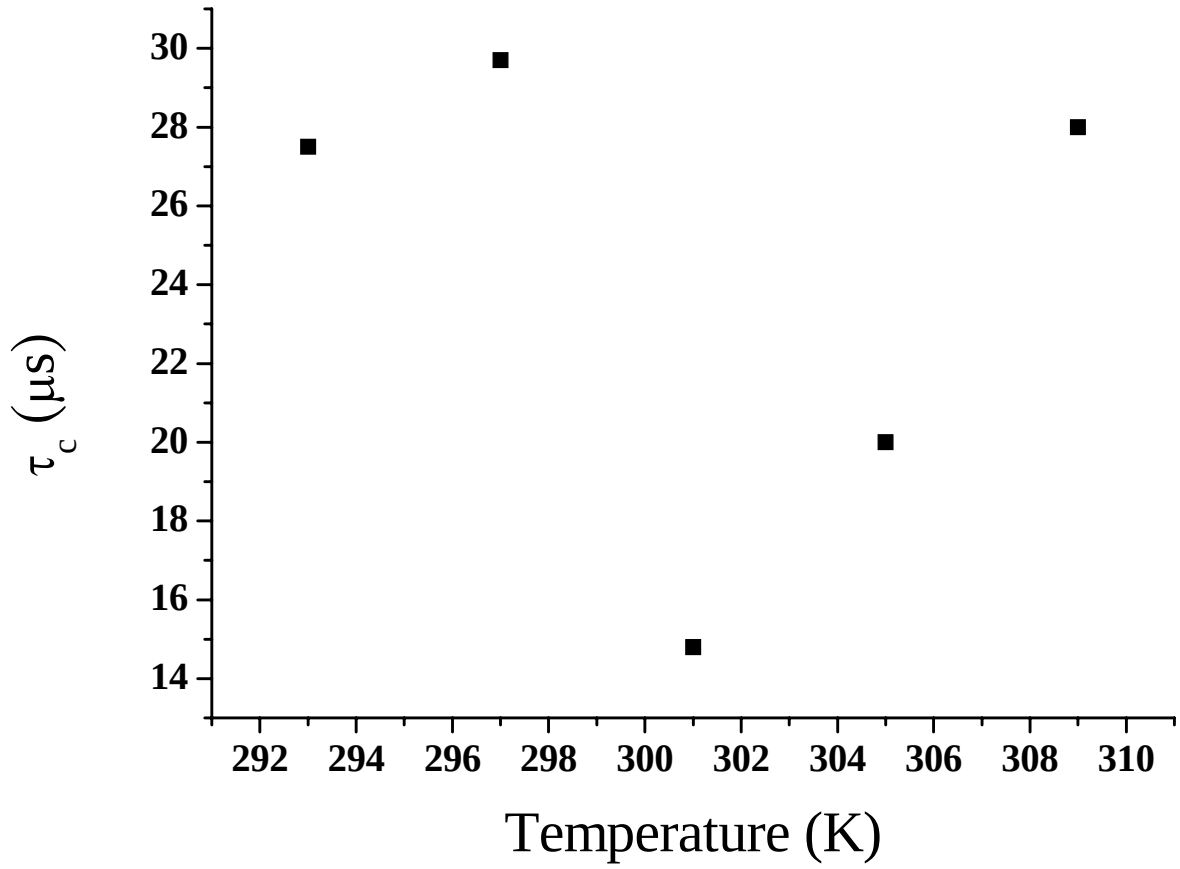



Figure 3a.

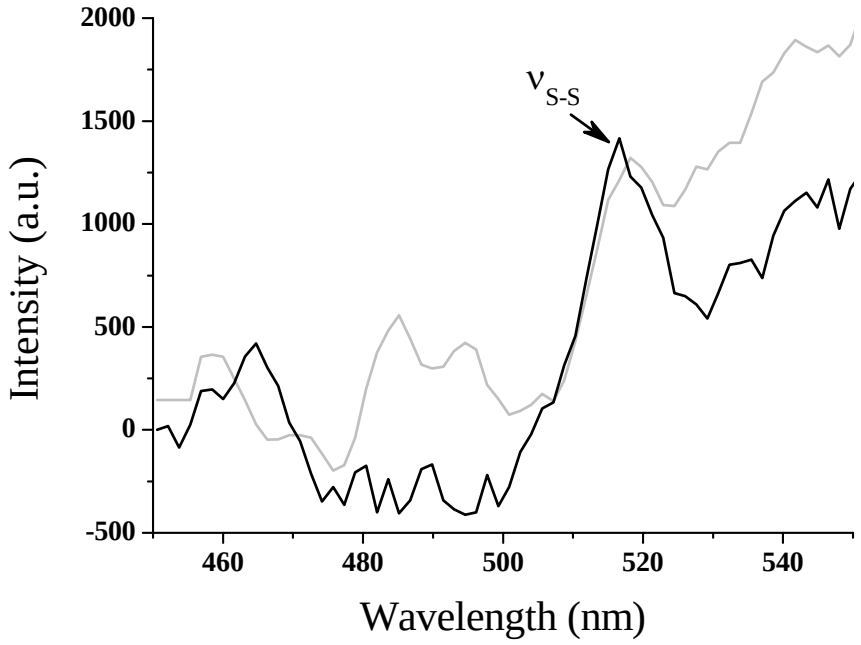


Figure 3b.

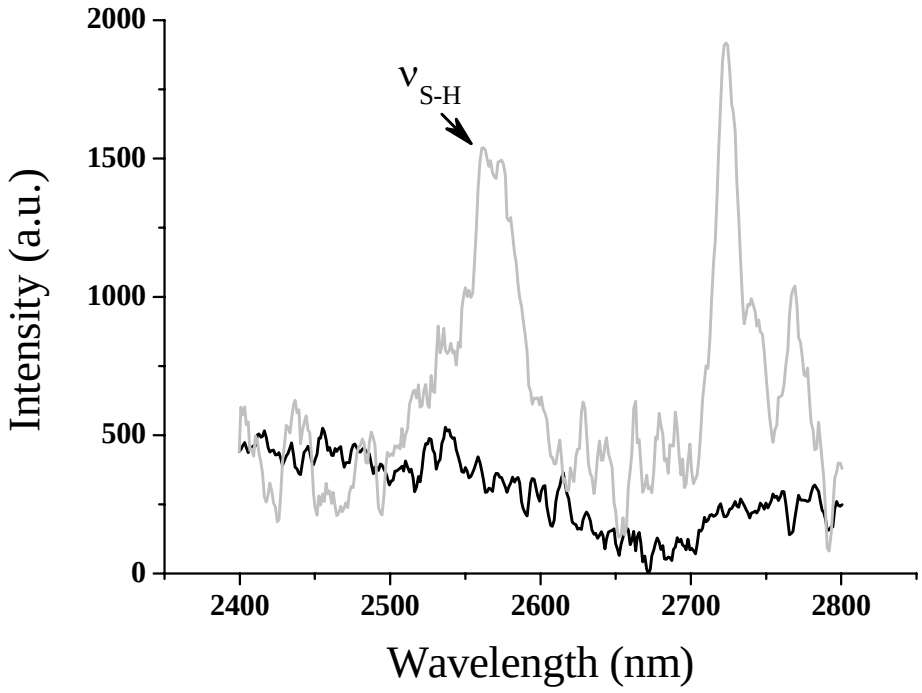
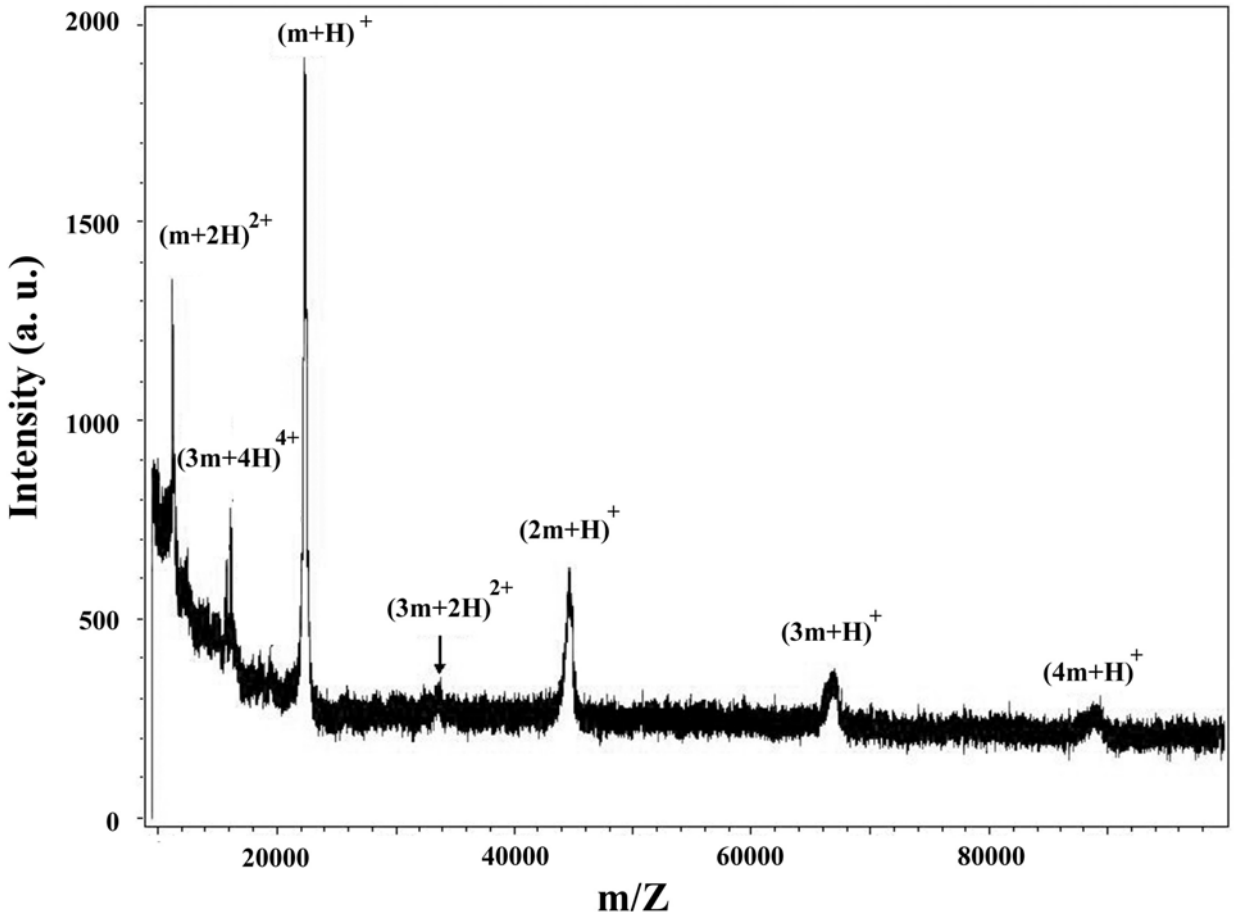

2000
1500
1000
500
0
Intensity (a.u.)
ν S-H
2400
2500
2600
2700
2800
Wavelength (nm)

Figure 4.


2000
1500
1000
500
0
Intensity (a. u.)
(m+H)+
(m+2H)2+
(3m+4H)4+
(2m+H)+
(3m+2H)2+
(3m+H)+
(4m+H)+
20000
40000
60000
80000
m/Z

**計畫成果自評：**

如前所述本計畫年度中我們已初步瞭解蛋白質摺疊與聚集之反應機制，並且已撰寫成兩篇學術論文送交國際期刊發表，其中已有一篇被接受且發表另一篇經審議後正在修改重審中。此外相關蛋白質摺疊與蛋白質自組織之研究亦有三篇論文為國際期刊接受或發表。其中更有一篇為 Applied Physics Letter 接受。成果極為豐富，相信在未來之計畫年度中應有更豐碩的成果。