

國立交通大學

應用化學系分子科學碩士班

碩士論文

重水中 L-苯丙胺酸的
雷射捕捉結晶化過程的研究

Studies on laser trapping-induced crystallization
dynamics of L-phenylalanine in D₂O

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中華民國一百零二年七月

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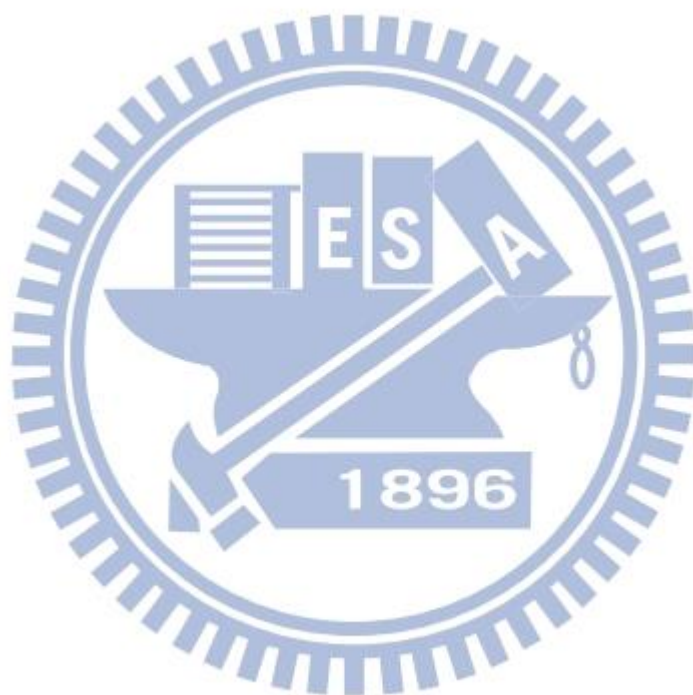
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摘要

過去十年間，我們將利用高倍率和高 NA(Numerical aperture)的物鏡所聚焦的 1064 奈米連續波長雷射光壓應用在甘胺酸、脯氨酸、絲氨酸等各種不同的基本胺基酸上並成功的證實雷射捕捉誘發結晶化的現象，我們命名為「雷射捕捉結晶化」。最近幾年間，我們成功在水溶液中把 L-苯丙胺酸利用雷射捕捉結晶技術結晶化並導致其無水結晶的生成，我們解釋無水結晶的生成現象是由於局部雷射加熱導致溫度超過轉變點 35-37°C 而產生脫水的結晶。

在這篇論文中，我們藉由控制雷射捕捉呈現出 L-苯丙胺酸在重水中的單水合和無水結晶。與水溶液不同，重水溶液中雷射加熱造成局部溫度的上升的效應還不足以達到轉變點。因此，無水結晶的生成可以確定是由雷射捕捉所實現的，並且我們發現結晶生成的機率和溶液的濃度及雷射極性十分

相關。完整的動力學及機構會以雷射捕捉下具有其特殊排列的團簇集合體及雷射捕捉在不同極性的雷射條件下對集合體中所引起的脫水現象的觀點來討論。



Studies on laser trapping-induced crystallization dynamics of L-phenylalanine in D₂O

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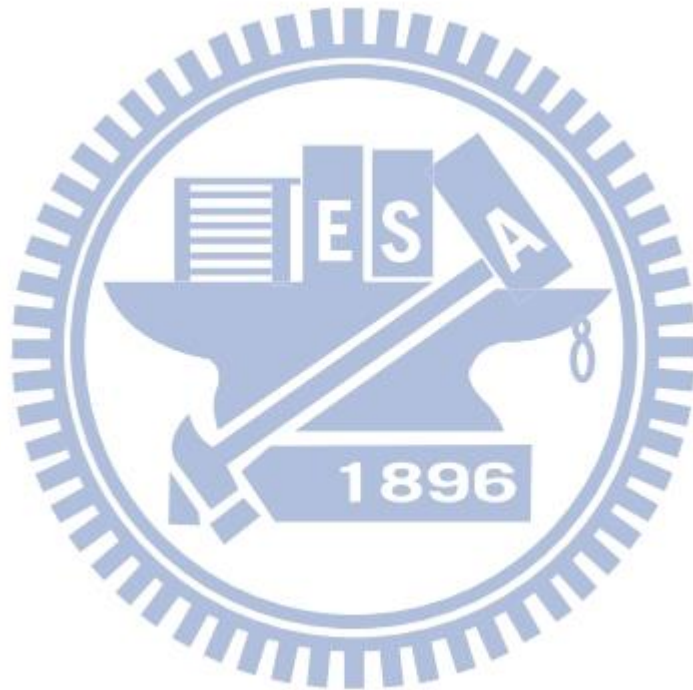
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Abstract

For the past decade, we applied radiation pressure of an intense CW laser beam of 1064 nm to various kinds of amino acids such as glycine, proline, serine, etc, and succeeded in demonstrating their laser trapping-induced crystallization, as we named this phenomenon “Laser trapping crystallization”. Most recently, we also succeeded in laser trapping crystallization of L-phenylalanine (L-Phe) in H₂O leading to the anhydrous crystal formation and explained that the anhydrous crystal formation was due to local laser heating over the transition point of 35-37°C.

In this thesis, I present pseudopolymorphism of L-Phe in D₂O leading to monohydrate and anhydrous forms controlled by laser trapping. Different from the H₂O solution, local temperature elevated by laser heating is too low to reach the transition point of L-Phe. Hence, the formation of anhydrous crystal was surely realized by laser trapping, and it was found that the formation probability strongly depends on solution concentration and laser polarization. The

entire dynamics and mechanism is discussed in view of formation of cluster assembly characteristic under laser trapping and laser trapping-induced dehydration in the assembly depending on laser polarization.



Acknowledgement

I am very happy to join this laboratory, big family, and “global village” during these two years. In these two years, I learned many new things and knowledge, and also cannot believe that I had a lot of chance to talk with foreign professor, visitors, and students. It was really an amazing and exciting experience. I will regard the time I experienced in NCTU as treasure in my mind.

At first, I am sincerely express my appreciation to my referees; Prof. Hiroshi Masuhara (National Chiao Tung University, NCTU), Prof. Wen-Sheng Chung (National Chiao Tung University, NCTU), Prof. Shinsuke Shigeto (National Chiao Tung University, NCTU), Prof. Atsushi Miura (National Chiao Tung University, NCTU), Prof. Yen-Ju Cheng, and Prof. Hsin-Yun Hsu (National Chiao Tung University, NCTU). They gave me a lot of advises for my works and master thesis; hence, I finished my thesis perfectly and successfully.

I deeply appreciate my supervisor Prof. Atsushi Miura, a respectable, responsible and resourceful scientist, who has provided me with valuable guidance in every stage of my master course. He kindly welcome me and teach me basic experiment knowledge when I enter the laboratory. Without his reminder, I cannot pass the master course smoothly. I will keep his teaching and guidance not only in my mind but also in the future study.

I shall extend my thanks to my co-advisors Prof. Hiroshi Masuhara, Dr. Teruki Sugiyama (Instrument Technology Research Center, ITRC), and Dr. Yuyama ken-ichi (National Chiao

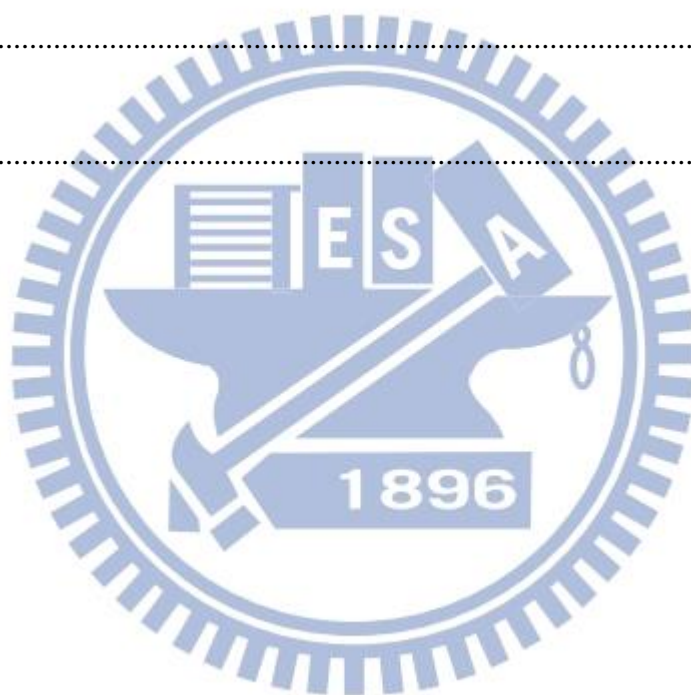
Tung University, NCTU) for their kind help and advice. Prof. Hiroshi Masuhara construct an international exchanging system in our laboratory, and I felt fortunately that I have many opportunity to get new knowledge and idea under this system and join his group. He also trained and taught me to think and consider by myself. I will keep this attitude in the future. Dr. Teruki Sugiyama and Dr. Yuyama ken-ichi helped me a lot not only in my master study but also in this thesis. They taught me a lot of new knowledge and idea related to my study. Without their comment, I cannot finish this study so quickly.

Last but not least, I would like to thank all my friends, family, especially my laboratory members of assistant Ms. Wen-Yu Lee, who always managed our school affair, my seniors Ping-Yu Hee, Jing-Ru Tu, Shun-Fa Wang, Wei-Yi Chiang, Ling-Ting Huang, Tzu-Wei Hsu, and Yan-Hua Huang, who kindly reply me when I have some problem on experiment and study, and my juniors Po-Yu Lin and Chun-Sheng Wu, who accompanied with me. With their encouragement and support, I really enjoy my master life.

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1. Introduction

1.1 Laser trapping

Laser trapping has been a powerful technique for trapping and manipulating micrometer-ordered particles in many kinds of research fields of chemistry, biology, and physics. The technique offered an accurate method to manipulate the particles from a few tens of nanometer to tens of micrometer size by focusing incident laser beam. We have applied this useful technique to L-Phe/D₂O solution in this work in order not only to control crystal pseudopolymorph but also to demonstrate laser trapping induced dehydration during the crystallization process.

1.1.1 History of laser trapping

Arthur Ashikin pioneered the field of optical trapping by introducing the laser in the early 1970s. He demonstrated optical force can trap micrometer-sized dielectric particles both in water and in air,¹ and found the gradient force and scattering force of radiant pressure by utilizing the single laser beam as “optical tweezers”.^{2,3} Furthermore, he and his colleagues also applied this technique to manipulate live bacteria and viruses later.^{4,5}

Chu and his colleagues also extended this technique to trap atoms, and he received the 1997 Nobel prize in physics along with Claude Cohen-Tannoudji and William Daniel Phillips by this

work of laser cooling and trapping neutral atoms.⁶ In fact, it was a very big breakthrough to control atom in 1 Å scale.

Nowadays, laser trapping has continued to find many useful applications in physics, biology, and chemistry with the size reduction of micrometer to nanometer,^{7,8} being developed to combine with single molecule microscopy technique.⁹

1.1.2 Principle of laser trapping

Laser trapping is due to tightly focusing a laser beam with an objective lens of high numerical aperture (NA). Due to the transfer of momentum from incident photons, a dielectric particle which near the focus spot will be trapped by the trapping force. The trapping force can be decomposed into two parts: gradient force and scattering force. The former is in the direction of spatial light gradient, and the latter is along the direction of light propagation. The scattering part is more familiar of this two, which can be thought as the photon pushing the particle along with the direction of light propagation, and scattering force is dominates for most conventional situations. The gradient force is caused from the fact that the dipole in the inhomogeneous electric field is affected by a force in the direction of field gradient. For stable trapping of objects, three-dimensional intense gradient force is necessary. The steep gradient force which moves the objects to focal region must be stronger enough to exceed the scattering force which makes the object away from the focal volume. This condition is provided when tightly focused laser is achieved by using the objective lens of high NA.

In developing a theoretical treatment of optical trapping, there are two limiting cases for which the force on a sphere can be readily calculated. When the trapped sphere is much larger than the wavelength of the trapping laser, the conditions for Mie scattering are ignored. Refraction of the incident light by the sphere corresponds to a change in the momentum transfer from the light (Fig 1.1).¹⁰ According to Newton's third law, an equal and opposite momentum change is generated for the sphere. If the refractive index is larger than the surrounding medium, the sphere will move to the focus point (Fig 1.2).¹¹

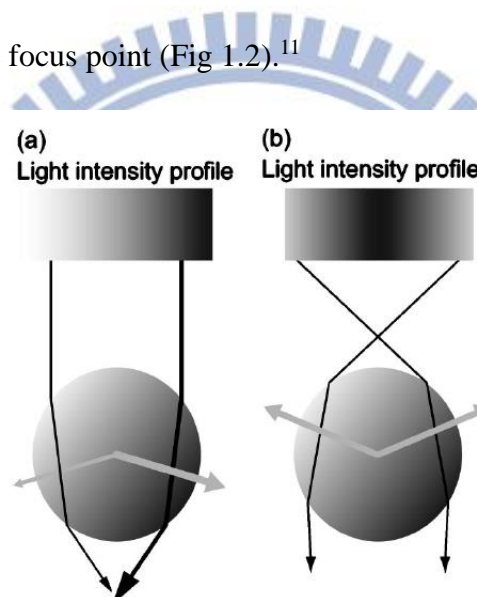


Fig. 1.1 Ray diagram shows difference of the direction of gradient force (gray arrows) induced by (a) unfocused and (b) focused light.

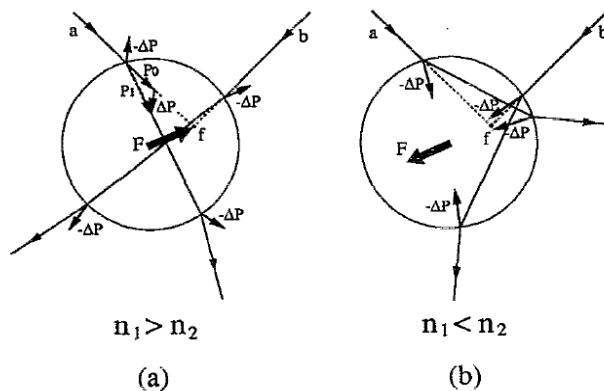


Fig. 1.2 Schematic drawing of the relationship between refractive indices of object (n_1) and medium (n_2) and the direction of gradient force.

In contrast, the direction of gradient force imparts to sphere is opposite when refractive index is lower than the surrounding medium.

On the other hand, Raleigh scattering cannot be ignored in the case of trapping of small spherical object which is much smaller than the wavelength of trapping light source. Under this condition the object is considered as a dielectric particle, i.e. point dipole. We need to consider the interaction between an electric field of the light and dipole moment of the particle. Gradient and scattering forces are corresponding to the first and second term of equation 1.1, respectively, where E is electric field, and B is magnetic field. An equation 1.2 depicts α which is the polarizability of a particle to be trapped, where r is the radius of the particle, and ϵ_2 is the dielectric constant of the surrounding medium. n_1 and n_2 are the refractive indices of the particle and the surrounding medium, respectively.

$$F = \frac{1}{2} \alpha \nabla |E|^2 + \alpha \frac{\sigma}{\sigma t} (E \times B) \dots \dots \dots (1.1)$$

$$\alpha = 4\pi\epsilon_2 r^3 \frac{(n_1/n_2)^2 - 1}{(n_1/n_2)^2 + 2} \dots \dots \dots (1.2)$$

As the high NA objective lens is employed, the trapping potential can be simplified and regarded as the equation (1.3). Once the trapping potential overcomes the Brownian motion whose energy should be $k_B T$, where k_B is the Boltzmann constant and T is the temperature in Kelvin, photon pressure makes it possible to control the object.

$$U = -\frac{1}{2}\alpha|E|^2 \dots\dots\dots (1.3)$$

Similarly, as in Ray optics, the condition for driving the object toward the focal region, the refractive index of the object should be larger than that of medium ($n_1 > n_2$)

1.1.3 Laser trapping-induced assembly of molecules

Different from single particle manipulation by laser trapping, application of this technique allowed scientists to investigate the interaction of numbers of particles such as colloids,¹² polymers, and membranes.¹³ Additionally, it can be applied to collect molecules. Indeed it has been demonstrated to assemble small particles of colloids and polymers^{14,15} to create their assembly which is as large as the focal spot size. Fig. 1.3 depict schematic representation of assembly formation of PNIPAM (Poly(*N*-isopropyl acrylamide)) that induced by focusing of trapping light source into the solution.¹⁵ Masuhara *et al.* have investigated the assembly formation of polymer molecules under photon pressure¹⁶⁻¹⁸ and its solvent dependence.¹⁹

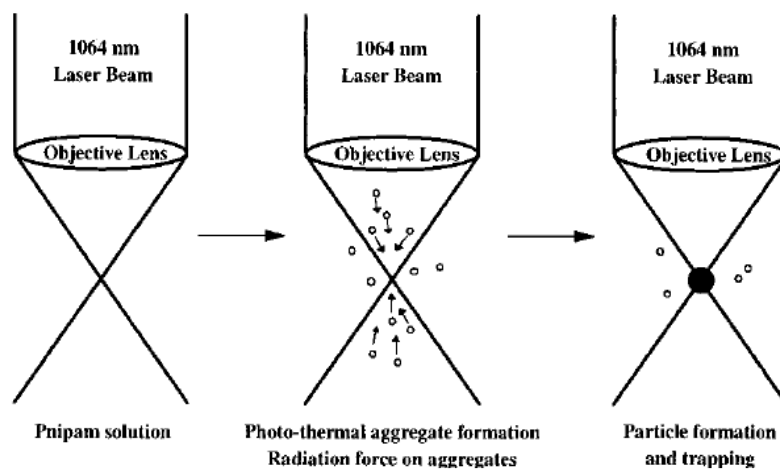


Fig. 1.3 Schematic view of PNIPAM assembly via photon pressure and phase transition.¹⁵

On the other hand, heat generation by absorption of focused light is inevitable in laser trapping. It induces Marangoni convection^{20,21} and enhances mass transfer²² that increases molecular transportation which should cooperate with photon pressure to collect molecules. As we can see in the phase transition of PNIPAN,¹⁵ we describe this matter in the last paragraph. Recently the convection flow under trapping could be realized in Fig. 1.4 by T. Uwada *et al.*²³

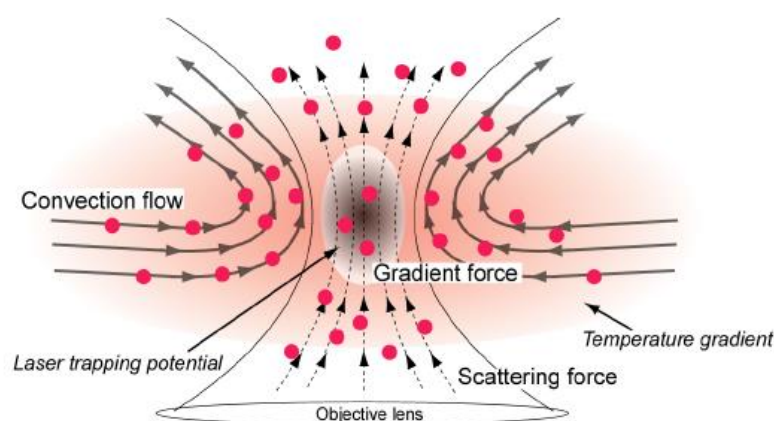


Fig. 1.4 Schematic picture of possible convection flow and trapped molecules brought up by Uwada *et al.*

Above mentioned observation of molecular assembly formations induced by laser trapping imply a possibility of more advanced molecular assembling, i.e. well-ordered molecular assembly such as crystallization should be possible. Tsuboi *et al.* confirmed the assembling of several amino acids by observing their Raman scattering spectra and backward scattering.²⁴ They explained that laser trapping induced assembly is probably due to trapping clusters of solute molecules. Lysozyme was first succeed in laser trapping-induced assembly under photon pressure, and crystallization was observed in few days after the further irradiation.²⁵ It reported crystallization could be induced by photon pressure causing protein aggregate. Besides, in the

same proteins, lysozyme, W. Singer applied laser trapping to induce the crystal growth²⁶ and investigated crystal growth directly under trapping condition.²⁷

1.2 Crystallization

We study crystallization mechanism which induced by laser trapping. Crystallization is closely related to our daily life such as the salt isolation from seawater and diamond formation in the deep earth under high temperature and pressure. Crystal provides molecule packing information and is used in wide fields of science and technology.

1.2.1 Conventional nucleation theory

Crystallization is a phase-transition phenomenon and also widely used as a purification method. Usually, solution for crystallization must be under supersaturation, and for achieving supersaturated solution there are many variable methods such as vapor pressure, temperature and pH valve. Crystallization process can generally be separated into two parts of nucleation and crystal growth processes. The birth of a new crystal is called nucleation: it indicates that molecule aggregate becomes larger than the critical size. Traditionally, the classical nucleation theory has been employed for the nucleation process, but it starts with tiny size and it is difficult to observe experimentally (Fig. 1.5).²⁸ Many papers have studied the nucleation and its mechanism in detail ^{29,30}. After nucleation, a subsequent process is known as crystal

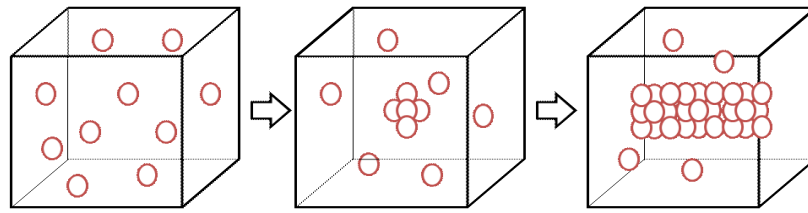


Fig. 1.5 Schematic drawing of nucleation, molecules start to pack with each other.

growth where nuclei grow larger. Molecules are continuously packing with each other in the regular ordering.

The process of crystallization also can be schematically illustrated by phase diagram (Fig. 1.6). The diagram is well interpreted with influential parameters of crystallization such as concentration and temperature. Under this condition, solution would be divided into three parts depending on the solution saturation. Once solution became highly saturated with higher free energy in the labile or metastable region, nucleation could take place, causing a reduction of free energy and the phase returned to the stable region. As mentioned later, we can suppose the dense liquid is regarded as precursor of crystallization in the intermediate state as seen in Fig.

1.7³¹.

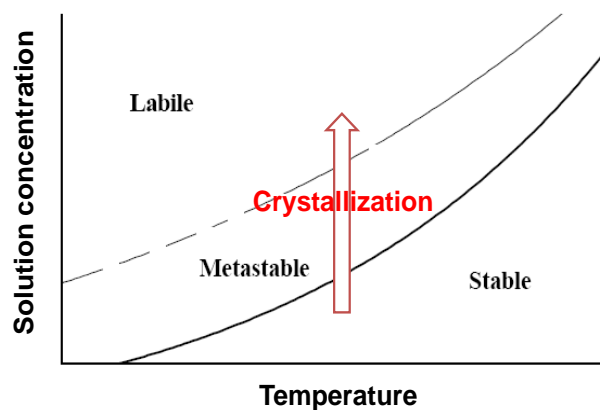


Fig. 1.6 Phase diagram showing the solubility depends on temperature and concentration.

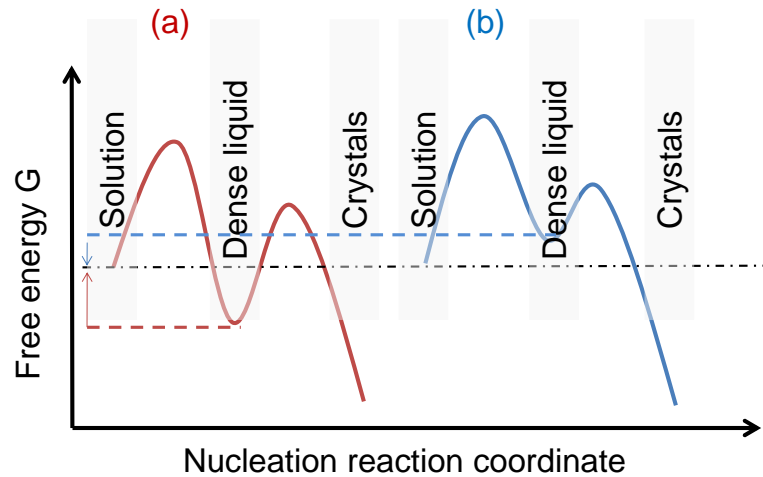


Fig. 1.7 Free energy diagram for possible crystallization processes. Curved depict phase transition from liquid, dense liquid as intermediate state, and crystalline phases. Black broken line indicates the free energy for liquid-liquid phase separation. Red and blue broken lines indicate the energy of droplet with (a) lower and (b) higher free energy than that of initial liquid phase.

1.2.2 History of biomolecular crystallization

According to the history of crystallization described by McPherson,³² it can be traced back to more than 170 years ago. The first published observation of crystallization was reported by Hunefeld on hemoglobin in 1840.³³ They reported the crystallization of blood of earthworm when it was pressed between two microscope slides. And later studies, Sumer and Stanley were awarded the Nobel Prize for chemistry in 1946 by isolation and crystallization of proteins and viruses.³⁴ In addition, it is also significant to analyze the structure of crystal to clarify functions of biomolecules. The first structural determination of biomolecule has done for vitamin B-12 in 1957 by D. C. Hodgkin by using its crystal.³¹ She received the Nobel prize for chemistry as the result of this research.

Crystallization has been studied for long time, however, its process is very complex and crystallization of some large biomolecules is still very difficult. In order to understand the fundamentals of crystallization to obtain better crystals, massive efforts were made on optimized crystallization and crystal growth of basic molecules such as small organic compounds and amino acids. Crystallization of amino acids, giving basic information to understand it, is still important works for protein crystallization. This is one of the reasons why amino acids were employed in this work. Although crystallization is still quite empirical, further understanding of crystallization can be expected.

1.3 Light-induced crystallization

Light-induced crystallization can be realized with various light through different mechanisms such as photochemical and nonphotochemical processes, where application of light source includes the uses of Xe lamp, CW laser or pulse laser at different wavelengths ranging from UV to NIR region.

There are conventional methods to crystallize such as Batch crystallization and vapor diffusion. Laser-induced crystallization has received much attention and development because it could regularly generate crystals, control the initial orientation giving different morphology of crystals, and finding novel crystal structures. Moreover, in this work, spatiotemporally control of crystallization and crystal pseudopolymorph could be achieved by laser trapping.

1.3.1 Photochemical reaction-induced nucleation

About the early work of the photochemical reaction-induced nucleation, John Tyndall has studied in a range of vapors and solutions in 1869,³⁵ and crystallization of lysozyme by conventional lamps also could be achieved.³⁶ In 2005, Okutsu *et al.* have reported photochemical reaction-induced crystal growth and morphology control of anthracene.³⁷ Furthermore, they have investigated photochemical reaction-induced nucleation of hen egg-white lysozyme (HEWL),³⁸ thaumatin,³⁹ and ribonuclease A,⁴⁰ From these results, the light with high energy is enough to cause ionization or create radicals and subsequent reactions inducing nucleation.

1.3.2 Non-photochemical laser-induced nucleation

Optically induced nucleation was discovered first by Garetz *et al.* in 1996⁴¹. In their work, supersaturated solution of urea was irradiated by 20 ns pulse of 1064 nm laser light with energy of about 0.1 J per pulse. It is considered as nonphotochemical reaction because the power and wavelength of light were not able to cause photochemical reaction. The result showed polarization dependent orientation of crystallite of urea where molecules were oriented along the incident light polarization. Authors suggested that this phenomenon was probably caused by the optical Kerr effect. Besides, following their discovery, most notably case, α - and γ - polymorphs of glycine crystals were induced from solution by circular and linear polarization of light, respectively; it was mentioned as polarization switching⁴². Polarization switching

could be made possible by the matching between packing arrangements of molecule and polarization of light. For example, α -glycine is composed of cyclic dimers. Meanwhile γ -glycine is composed of helical chains. Recently, polarization switching was also reported in case of L-histidine.⁴³

Instead of Kerr effect mechanism, other nonphotochemical laser-induced crystallization method had been also developed. For example, femtosecond laser induced crystallization through bubble formation,⁴⁴ single pulse crystallization, mechanism for the effect involves the isotropic electronic polarization of cluster⁴⁵ and this work which is laser trapping crystallization.

1.3.3 Laser trapping crystallization

Sugiyama *et al.* demonstrated glycine crystallization induced by laser trapping in 2007.⁴⁶ It was the first observation of the crystallization only by focused irradiation. They named this method as “laser trapping crystallization”. It was not only a new application of laser trapping but also novel methodology of crystallization.

In general, where and when crystallization took place is not clear, but now it is always observed at the focal spot via laser trapping within a few minutes. Moreover, they reported spatially-controlled crystal growth⁴⁷ and molecular orientation in crystal, as direction of crystal growth was directed toward laser spot and different polymorph of glycine crystal were prepared by adjusting laser power.⁴⁸

Initially, laser trapping crystallization was reported for supersaturated glycine solution.

Usually it is impossible to crystallize molecules in unsaturated solution, but they can observe crystallization even in unsaturated solution by laser trapping crystallization.⁴⁹ It suggests that local concentration increase to supersaturated value in the focal spot due to laser trapping of the clusters, leading to crystallization.

Besides, laser trapping crystallization showed not only spatiotemporally controlled crystallization but also very interesting photon pressure-induced phenomenon; large liquid droplet formation. Yuyama *et al.* demonstrated millimeter-sized dense liquid droplet formation of glycine. They observed the droplet formation by focusing the trapping laser to the solution/substrate interface.⁵⁰ Moreover, glycine crystallization was observed immediately after when the focus of trapping laser moved to the air/solution interface. It implies higher-concentration droplet formation, i.e. local concentration elevation due to the photon pressure, and they concluded that the dense droplet would be a precursor of the crystal.

1.4 Purpose and goal

We are interested in molecule assembly formation and crystallization induced by photon pressure. Since laser trapping crystallization just succeeded in limited number of molecules and its behavior is not totally clarified, more extension of experiments involving other amino acids have been tried till today. Indeed the crystallization of bio-molecules and crystal structural control is quite important technique. We need to understand dynamics and mechanism of molecular crystallization and transformation of crystal polymorphs under photon pressure to

establish this method as a general crystallization technique.

In this work, we intend to investigate the dynamics and mechanism of dehydration and crystallization and influence of different concentration and polarization. This is the new phenomenon and would be the indication in the crystal selection or filtration. By completing and realizing these processes, we may control crystal polymorphism by changing laser polarization, wavelength, and power.



2. Experimental

2.1 Material

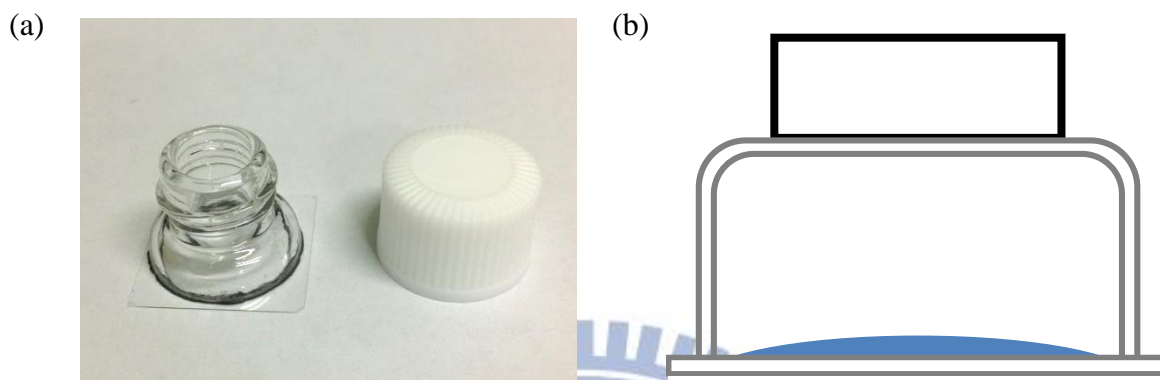


Fig. 2.1 Pictures of the container. (a) Cut glass vial, (b) Schematic drawing and solution condition

L-phenylalanine (L-Phe; > 99 %) in the form of white powder was obtained from Sigma-Aldrich and used as a target sample without any further purification. D₂O (> 99 %) was also obtained from Sigma-Aldrich and used as a solvent after filtration with a syringe filter. A home-made closed glass container (Fig. 2.1 (a)) which was made by gluing (Shin-Etsu Silicone, 1 component RTV) a cut glass vial (Nichiden-Rika glass) on a cover glass (18×18 mm) was used as experiment container. The glass container was sunk in detergent at least for one day, and then washed by deionized water repeatedly. After cleaning of container, the glass substrate became highly hydrophilic which made sample solution form a thin layer and homogeneously spread on the bottom of glass substrate easily (Fig. 2.1 (b)). A portion of L-Phe solution (15μL) was poured into glass container, and the thickness of initial solution layer was about 120-160 μm.

2.1.1 Sample preparation

Concentration of L-Phe solution is 17.5, 20, 22.5, 25, 27.5, and 30 mg in 1g D₂O. There is no literature about solubility of L-Phe in D₂O, but we confirmed 22.5 and 27.5 mg are unsaturated and supersaturated solutions, respectively. Hence, saturated solution may located at about 25 mg/1g D₂O. Here we used deuterated water as solvents to suppress temperature elevation due to trapping laser absorption. In order to prepare stable supersaturated solution of L-Phe, L-Phe powders are completely dissolved into D₂O by vigorous shaking at 60°C using a bio-shaker (TAITEC, BR-21UM), and then the isotropic solution is slowly cooled down to room temperature (~25°C). Whole process can be achieved in one day. Before laser trapping crystallization experiments, we confirmed that no spontaneous crystallization occurs for 1 to 7 days. This aging time is much longer than the laser trapping experiments for 30 min, and then crystallization induced by 30-min laser irradiation should be due to local concentration increase by laser trapping. This is also supported by wide range observation using a CCD camera for the formed crystal, which details are described later.

2.1.2 Pseudopolymorph

Phenylalanine (C₆H₅CH₂CH(NH₂)COOH) is one of the twenty common amino acids. It contains benzene ring in its main framework. Chemical structure of phenylalanine is depicted in Fig. 2.2. L-Phe molecules almost completely exist as a zwitterion in the aqueous solution,

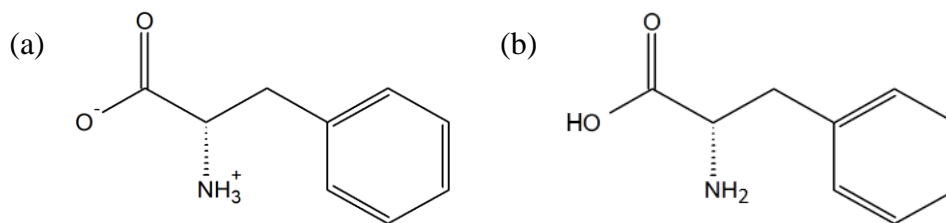


Fig. 2.2 Chemical structure of phenylalanine: (a) zwitterionic and (b) neutral form

when pH is equal to about 5.48 of the isoelectric point. In the D_2O solution, H atoms on carboxyl and amino groups can be replaced by D atom according to existence probability of H and D in the solution and the pD value is estimated by adding 0.4 to the pH value.⁵¹ Comparing with D-phenylalanine, L-phenylalanine cannot be produced by organic synthesis and also an electrically neutral and nonpolar amino acid which coded for by DNA.

It is well known that there are two kinds of pseudopolymorph of monohydrate and

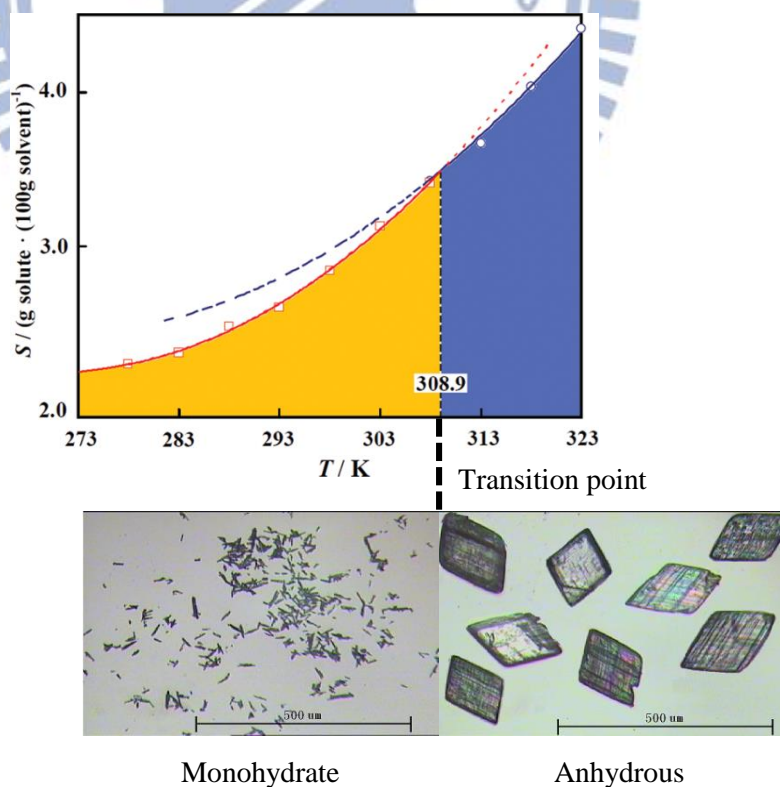


Fig. 2.3 Solubility diagram of monohydrate and anhydrous crystal

anhydrous L-Phe as shown in Fig. 2.3.^{52,53} Pseudopolymorphism is the ability of a crystal to exist in more than one form or crystal structure, in which solvent molecules are included as an integral part of the structure. Different pseudopolymorphs generally show different solubility, dissolution rate, mechanical behavior, stability and bioavailability from their unsolvated counterparts. For L-Phe, temperature is known to be the critical parameter to determine the crystal pseudopolymorphism, and temperature of the transition point is about 35-37 °C.^{52,54} Monohydrate form, needle-like crystal, which contained water molecules is the spontaneously formed crystal at room temperature, and anhydrous form, plate-like crystal, which without water molecules inside is usually generated above 35-37°C instead of monohydrate crystal. That is to say, spontaneous monohydrate crystal is formed under solution evaporation, and we cannot get anhydrous crystal spontaneously at room temperature.

2.2 Optical setup

Fig. 2.4 schematically shows a microscope and optical setup used in laser trapping crystallization study. Microscope setup is based on an inverted microscope (Olympus, IX71). Room temperature and humidity were controlled to be around 24~26°C and 50~60%, respectively. A near-infrared 1064-nm continuous-wave Nd:YVO₄ laser (Coherent, Matrix CW) with 1.1W was used as a trapping light. Three kinds of laser polarization was generated by adjusting quarter wave plate. In order to check the solution height, linearly polarized 632 nm

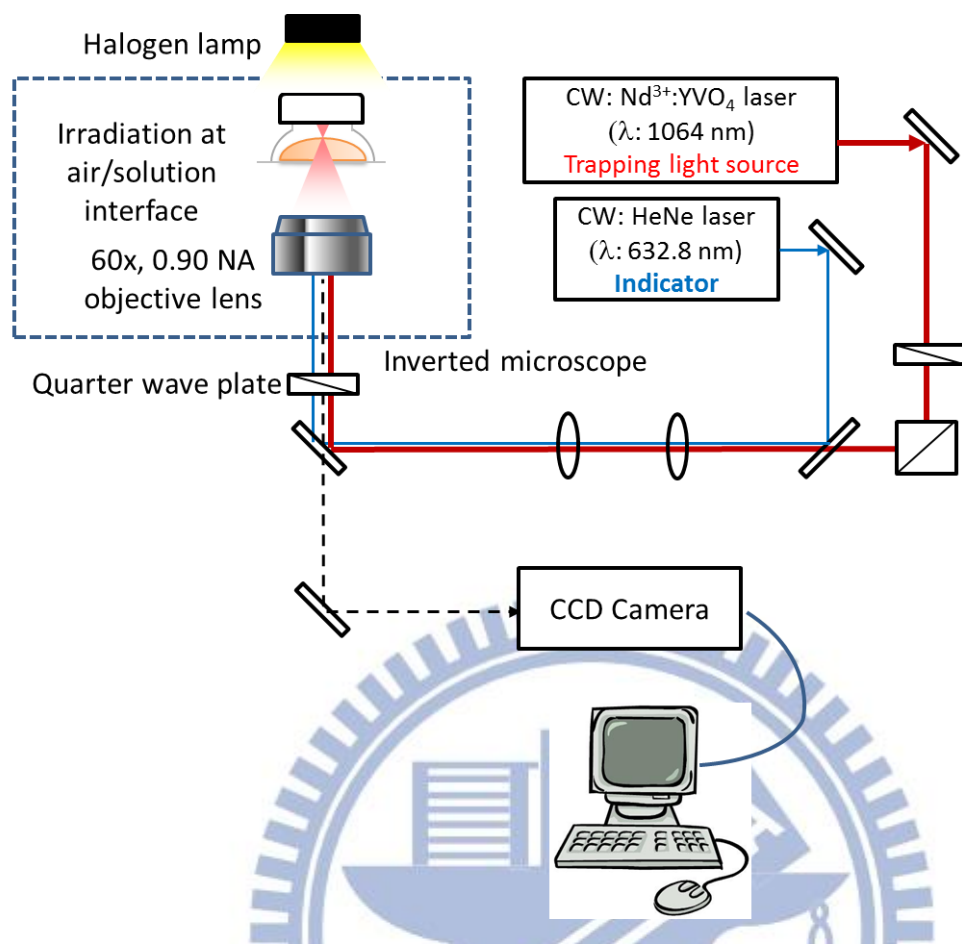


Fig. 2.4 Schematic diagram of optical set up of laser trapping crystallization system

HeNe laser was used as an indicator. The trapping laser and indicator were introduced into the microscope and focused at the air/solution interface through a 60x objective lens (N.A. 0.90). In order to achieve optimal trapping condition, trapping laser light was expanded and collimated to fully use pupil diameter of a microscope objective lens (~8 mm). Finally, the crystallization behavior was observed by CCD video camera.

2.3 Estimation of local temperature elevation by laser heating

Under laser trapping condition, temperature elevation due to absorption of solvent and

molecule cannot be avoided. We should consider it since temperature change affects physical property and chemical reactivity of molecules in the solution especially in L-Phe solution. In general, temperature elevation causes increasing solubility of solute molecule and decreasing supersaturated value (SS). Furthermore, it will cause crystal structure transformation in the case of L-Phe. However, temperature elevation not only enhance evaporation of solvent and increase of supersaturated value simultaneously, but also affect the crystal pseudopolymorphism. Thus, we need to estimate temperature elevation due to the absorption of trapping laser light at 1064 nm quantitatively.

Investigations of temperature increasing under laser trapping condition have been reported. In the case of 1064 nm CW YAG laser, Fischer reported that $\Delta T/\Delta P \sim 5$ K/W by thermal handing of langmuir-monolayers at the air/water interface.⁵⁵ Here T and P represent temperature in Kelvin and irradiated laser power in Watt. Schmid and Tromberg reported ~ 8 K/W and 10-14.5 K/W of temperature elevation of water, respectively.^{56,57} Ito also reported temperature elevation at the focused volume degree difference for ethylene, ethanol, and water as 62 ± 6 , 49 ± 7 , and 23 ± 1 K/W, respectively, upon laser trapping in small domain of solution based on the diffusion coefficient of fluorescent molecules determined by Fluorescence correlation spectroscopy.⁵⁸ Usually, in order to avoid such temperature elevation, we used D₂O as a solvent in our study. Due to over- and combination-tones of O-H vibration, laser beam is absorbed by solvent and causes high temperature in H₂O solution. On the contrary, O-D stretching of D₂O show little

absorption band at 1064 nm.

Here heat is generated by irradiated laser light that was absorbed by solvent and solute molecules. First, we checked the absorption by solvent and solute molecules on the basis of Beer-Lambert's Law,

$$I = I_0 e^{-\alpha l} = I_0 10^{-abc} \dots\dots\dots (2-1)$$

where I_0 and I are the intensity of incident and transmitted light, respectively. α and l are absorption coefficient and optical path length. Absorption also can be defined based on molar absorption coefficient ϵ , optical length b , and concentration c . For convenient, equation 2-1 can be rewritten to equation 2-2 which shows the exponential curve.

$$T(\%) = \frac{I}{I_0} = A \cdot e^{-\alpha L} \dots\dots\dots (2-2)$$

Fig. 2.5 shows the optical path length dependent transmittance change of H₂O, D₂O, L-Phe/H₂O, and L-Phe/D₂O aqueous solution measured at 1064 nm. All the absorption and transmittance spectra were measured by absorption spectrophotometer (JASCO, V-600). As depicted in Fig. 2.5 optical path length dependent exponential decrease of transmittance was observed for all samples. Deuterated solvents showed smaller diminution of transmittance than that of H₂O. It indicates that deuterated solvents absorb less 1064 nm light and, as a result, temperature elevation is smaller than others. Therefore we decided to use deuterated solvent to prevent temperature elevation in this research. In order to ensure the temperature elevation of

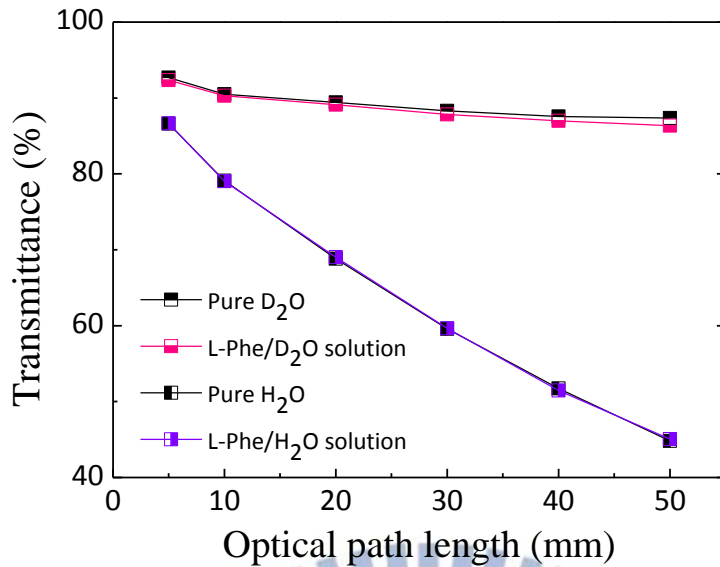


Fig. 2.5 Optical path length dependence of transmittance of (a) D₂O and H₂O, (b) H₂O and L-Phe/D₂O solution

L-Phe in D₂O, we measured and compared with L-Phe/H₂O again, and it shows almost the same results correlated to Pure D₂O and H₂O. By fitting the exponential curve (eq. 2.2) based on the least square method, absorption coefficients of each solvent are as follows; H₂O is $\sim 14.5 \text{ m}^{-1}$, D₂O is $\sim 1 \text{ m}^{-1}$, and L-Phe in D₂O solution is $\sim 0.9 \text{ m}^{-1}$.

Figure 2.6 shows the FCS works which is done by Ito,⁵⁸ and it shows linear correlation between temperature elevation coefficients and ratio of extinction coefficient and thermal conductivity. According to this figure, we calculated the temperature elevation and summarized the data in table 2-1. From these results, absorption coefficient of L-Phe/D₂O and L-Phe/H₂O solutions are almost the same as deuterated water and water, respectively, so that the contribution of L-Phe molecule in absorption of L-Phe/D₂O solution can be ignored. However, temperature elevation can be ignored in this study.

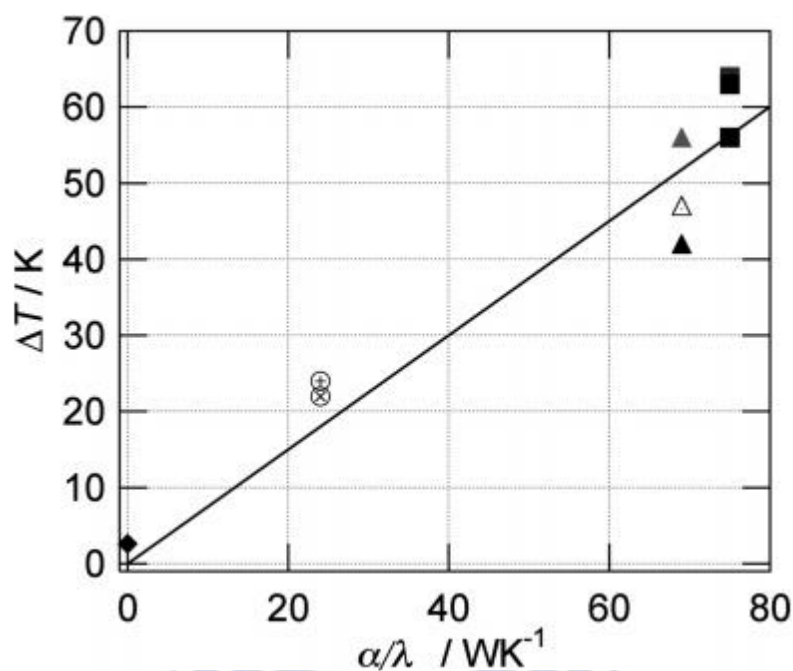


Fig. 2.6 Plot of the temperature elevation coefficients, $\Delta T/\Delta P$, as a function of the ratio of the extinction coefficient of the solvents, α , to the thermal conductivity of the solvents, λ .

Sample	α , absorption coefficient (m^{-1})	λ , thermal Conductivity [$\text{W m}^{-1}\text{K}^{-1}$]	Temperature elevation ($\Delta T/P$)
H ₂ O	14.5	0.59	~23
D ₂ O	1.2	0.59 (H ₂ O)	~2.6
L-Phe/H ₂ O solution	14.5	0.59(H ₂ O)	~23
L-Phe/D ₂ O solution	1.4	0.59 (H ₂ O)	~3

Table 2-1 Absorption coefficient (1064 nm), thermal conductivity and estimation of temperature elevation by irradiation (1064 nm) in different solvent

2.4 Crystal formation of L-phenylalanine in H₂O

As mentioned above, Yuyama *et al.* recently succeeded in crystallization of anhydrous form of L-Phe in H₂O (Fig. 2.7). In their works, the formation of anhydrous crystal was induced by laser heating in all the concentration (20, 22.5, 25, 27.5, 30 mg/1g H₂O; saturated solution is 30mg/1g D₂O) even in unsaturated solution. That is to say, laser irradiation generates

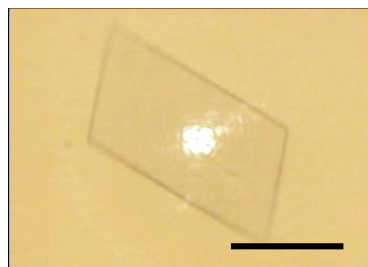


Fig. 2.7 Laser irradiation causes high temperature elevation enough to exceed the transition point, resulting in the anhydrous crystal formation.

high temperature elevation (20°C) due to the absorption of 1064-nm trapping light source, and it is enough to exceed the transition point, resulting in the formation of anhydrous crystal. It is surprising that crystal can be generated and grew up in unsaturation solution. In the case of glycine, crystal dissolved soon after crystallization. It is reasonable that concentration near the focal spot will be unsaturation after crystal nucleation, and crystal will dissolve under unsaturation. Not only that, but they also showed the results of different crystal growth rate by controlling the power of laser. The diameter of focal spot is about 1.4 μm ; in general, it is difficult to explain why the size of anhydrous crystal exceeded 100 μm under unsaturation condition. It may imply some mechanism or phenomenon enhanced the trapping efficiency. They suggested this process by the way of the laser light can be propagated to the surrounding of crystal by flat and thin L-Phe crystal. Through the L-Phe crystal, laser collect the clusters more efficiently. Therefore, even under unsaturation crystal growth is available for laser trapping. However, it is surprising that they illustrate the ability of collection, nucleation and crystal growth induced by laser trapping.

3. Results and discussion

3.1 Crystal formation of L-phenylalanine in D₂O

The reference or report about L-Phe in D₂O have not been clearly studied yet. Therefore, it is of great interest in laser trapping crystallization of L-Phe in D₂O. We did ten experiments for each concentration and repeated ten experiments few times to check showing same results every time. These results enable us explain the phenomenon by data and continue the further experiments. During the laser irradiation, crystal formation was observed for all samples within 30 minutes. Depending on the sample concentrations two kinds of crystal shapes of plate-like or needle-like form were prepared (Fig. 3.1). If laser irradiation is cut off immediately after the first crystal formation, laser irradiation always leads to one crystal formation. However, further laser irradiation after the crystallization sometimes provides another crystal form, especially in supersaturated solution. This result indicates that each crystal formation competitively takes place.



Fig. 3.1 Anhydrous and monohydrate crystal forms of L-Phe. (Left; anhydrous (plate-like), Right; monohydrate (needle-like))

Fig. 3.2 and 3.3 show the snapshots of process of two kinds of L-Phe crystal generation and growth at the air/solution interface, which was captured from experiment video. In the Fig. 3.2, one seed crystal was always generated at the focal spot and trapped by the focused laser after few minutes of laser irradiation. Few hundred micrometer sized crystal growth was

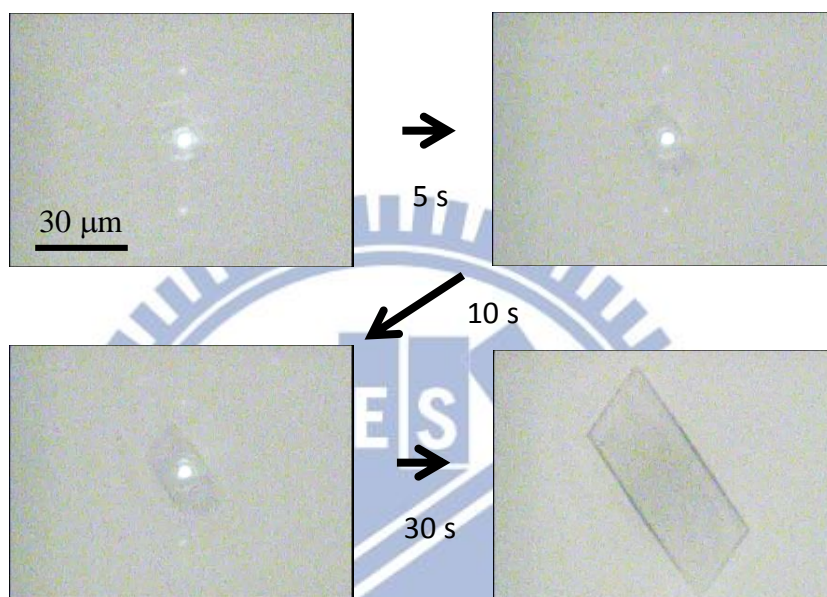


Fig. 3.2 Laser trapping-induced plate-like crystal formation.

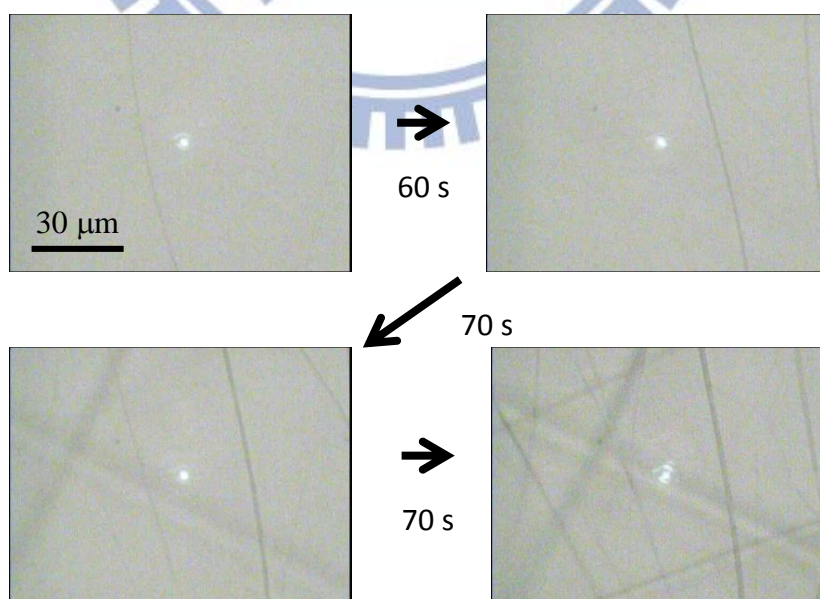


Fig. 3.3 Laser trapping-induced needle-like crystal formation.

followed by further laser irradiation. In Fig. 3.3, the needle-like crystal was almost generated not at the focal spot, and became more and more by further irradiation. Position of needle-like crystal formation is a few micrometer to a few tens of micrometer size from the focal spot depending on the solution concentration. The plate-like crystal was sometimes identified at the focal spot, after generation of many needle-like crystals. In order to confirm needle-like crystal is laser induced one, we check the spontaneous crystal, laser induced crystal, and needle-like crystal distribution under a microscope as shown in Fig. 3.4. Compared with 3.4 (a) and 3.4 (b), spontaneous crystallization takes place everywhere, and laser induced crystallization just generates around the focal spot. We can see more clearly from 3.4 (c), where laser induced needle-like crystals only formed 1.0 μm from the focal spot. It illustrates our plate- and needle-like crystals are generated by laser.

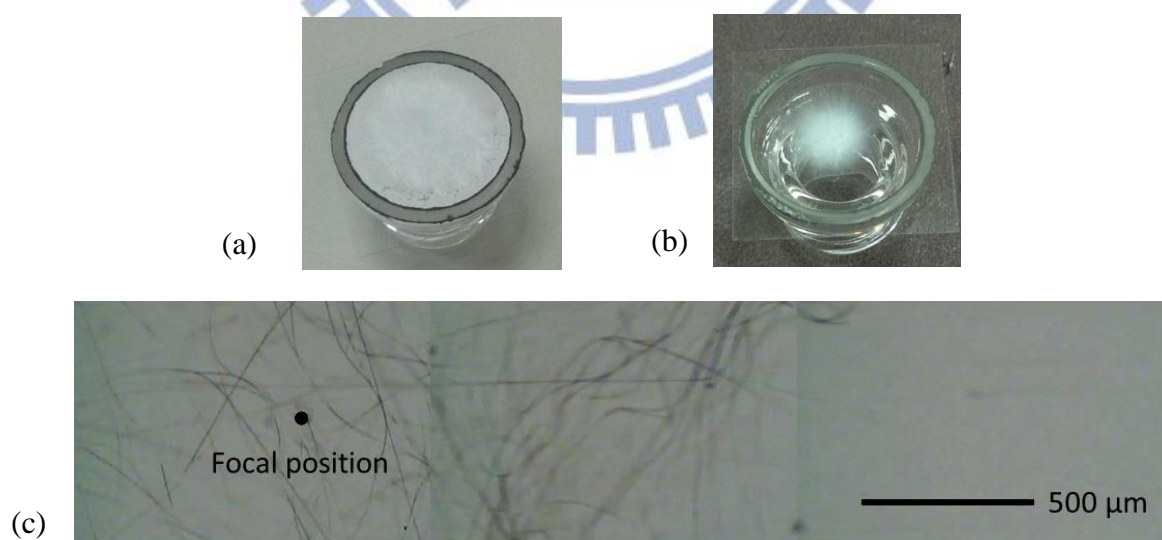


Fig. 3.4 Formation of needle-like crystals. (a) spontaneous crystallization, (b) laser induced crystallization, (c) laser induced needle-like crystal distribution under a microscope.

In our study, crystal pseudopolymorphism is clarified by shape which mentioned in H₂O case. Chemistry properties of D₂O is very similar to that of H₂O, so we believed that results will be similar to that of H₂O. In order to confirm two different pseudopolymorphism, we did FTIR measurement for each crystal form. Fig. 3.5 and 3.6 show the FTIR spectrum of two kinds of crystal we obtained from the experiments. We did six samples for each form, and all the spectra showed good consistency reflecting to each pseudopolymorph. There are some different bands between these two spectra at 2150 cm⁻¹, 1825 cm⁻¹, 850 cm⁻¹, and 650 cm⁻¹. Considering previous reports concerning pseudopolymorphism in H₂O, we suggest that plate-like and needle-like crystal are ascribed to be anhydrous and monohydrate crystal, respectively. Thus, I examined pseudopolymorphism by laser trapping from the morphology of the formed

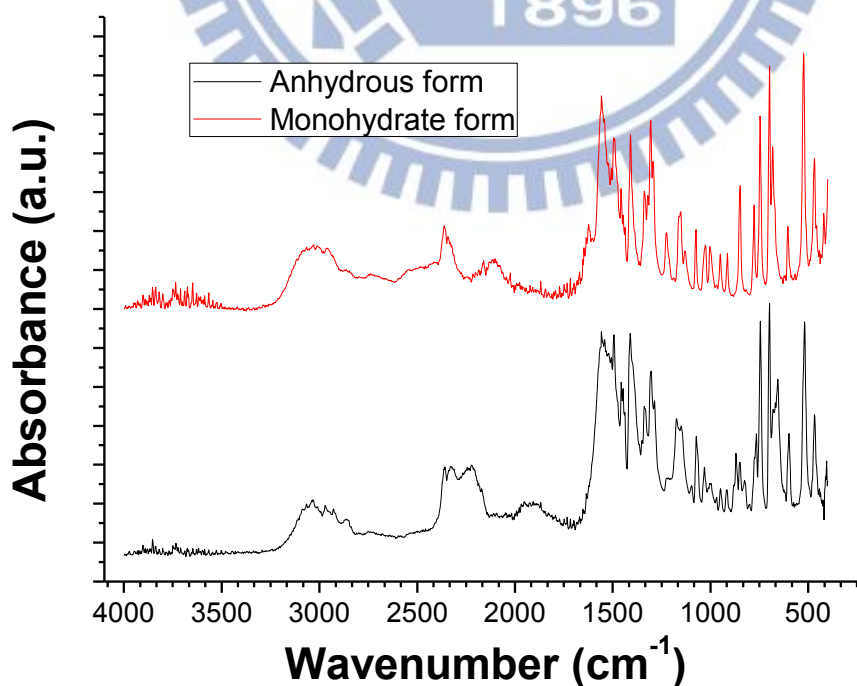


Fig. 3.5 FTIR spectra of two different crystal form of anhydrous and monohydrate, respectively.

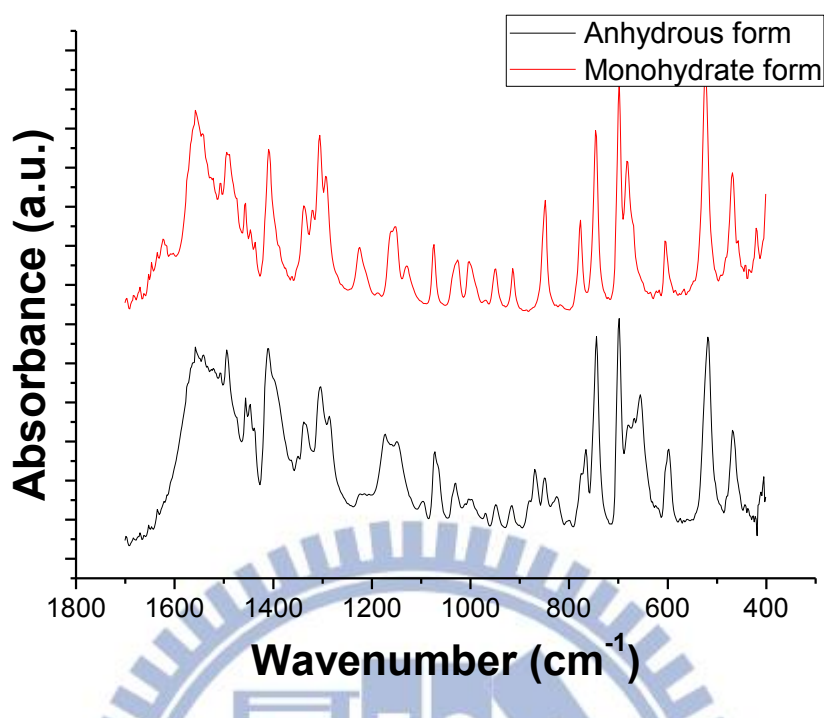


Fig. 3.6 FTIR spectra of two different crystal form of anhydrous and monohydrate at 400-1700 cm^{-1} , respectively.

crystal observed in the CCD images.

Monohydrate crystal is always prepared spontaneously at room temperature, so we are surprised that anhydrous crystal formed in the solution. As mention above, laser heat caused temperature elevation enough to exceed the transition point 35-37°C in H_2O , and gives the anhydrous crystal. In our works, temperature elevation was suppressed by using D_2O as a solvent compared to that of H_2O (Table 2-1), so we should consider the mechanism and process in other way.

3.2 Pseudopolymorphism: Concentration dependence

In our experiment, it is too difficult to trap the monomer, so liquid-like cluster is the trapped target which is spontaneous aggregation of L-Phe molecule and D₂O solvent due to concentration fluctuation. Size and amount of liquid-like cluster strongly depends on solution concentration, and it becomes more with concentration increase. We confirmed that the spontaneous crystallization at room temperature always provides the monohydrate crystal, 2.2 and 2.7×10^{-2} wt% are unsaturated and supersaturated solution, and the probabilities of anhydrous and monohydrate crystal formation at room temperature in different concentrations. After laser irradiation, either supersaturation or saturation even in unsaturation were observed monohydrate and anhydrous crystal formation in all sample within 30 minutes depending on the solution concentration, respectively. I summarized all the experiment results in Fig. 3.7. The

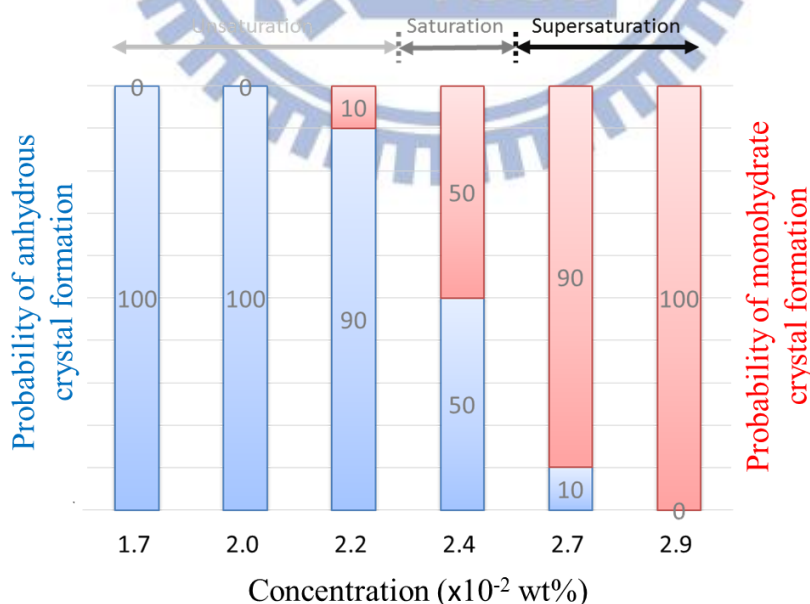


Fig. 3.7 Probability of plate-like crystal formation (blue line) and spontaneous crystallization (red line) depending on concentration.

probabilities of anhydrous and monohydrate crystal formation are calculated by the number of the crystal form prepared firstly after starting laser irradiation, and the irradiation was often turned off immediately after the crystal generation. Compared with spontaneous crystallization, our crystallization time is much shorter. It confirmed that all the crystals were induced by laser, and they were not spontaneous one even in supersaturation. The probability of anhydrous crystal formation became clearly high at the low concentration, it indicates anhydrous crystal formation is dominant in the solution. At high concentration, laser induced spontaneous crystallization occurred easily in the solution, but anhydrous crystal was still observed at 2.7×10^{-2} wt%. These results are much different from results in H₂O, which indicates the pseudopolymorphism were induced in different process again.

Under supersaturation, it shows laser enhanced spontaneous crystallization of monohydrate crystal always takes place in the solution, but one anhydrous crystal was still formed in 2.7×10^{-2} wt%. It means some competition may occur between monohydrate and anhydrous crystal formation, and also illustrates the ability of polymorph controlling and dehydration induced by laser as described later. As shown in Fig 3.8, initial solution of 2.9×10^{-2} wt% is full of liquid-like clusters and these liquid-like clusters are efficiently trapped by laser leads to the aggregate of liquid-like cluster in the focal spot like stage (i). Through the liquid nucleation, it goes to the stage (ii) where aggregate of liquid-like clusters is transformed to assembly of liquid-like clusters. In the stage (ii), assembly of liquid-like clusters starts to

become well-ordered, higher concentration and less solvent molecules. Assembly of liquid-like clusters which is trapped at focal spot enlarge the trapping potential and enhance trapping force, and then make high concentration propagating to the outside of focal spot and entering stage (iii). Here, we propose “laser pinning effect.” The alignment of molecules in the focal spot is reflecting to the polarization of laser, and the effect decrease with the distance from the focal spot. The alignment pinned by laser in the focal spot is not suitable for monohydrate crystal formation. As a result, monohydrate crystal always form not at the focal spot, but monohydrate crystal formation is achieved when molecular alignment become disordered far away from focal spot. On the other hand, molecules pinned by laser in the focal volume perform the large-linked molecules or clusters which is the precursor of anhydrous crystal. As local concentration is

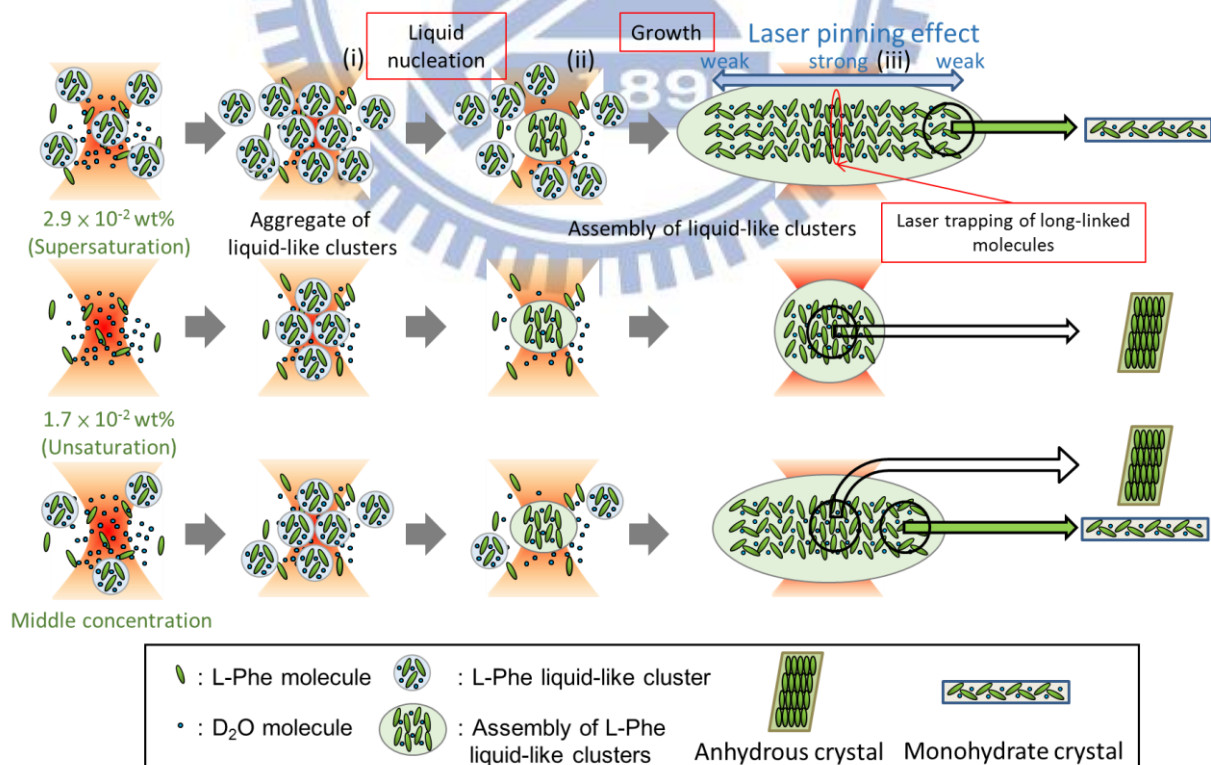


Fig. 3.8 Crystallization process of two crystal pseudopolymorphism under different solution concentration.

increased by laser trapping, the distance between L-Phe molecules becomes closer and the intermolecular interactions between L-Phe molecules becomes prominent. As a result, L-Phe molecules themselves directly linked with each other through relatively strong hydrogen bonds, and it causes local dehydration where strong interaction excluded the solvent, leading to anhydrous crystal formation. As described in section 2.4, dehydration in H₂O is induced by laser heating which is enough to exceed the transition point 37°C. It is quite different mechanism between these two, which one is due to temperature elevation and another is because of intermolecular interaction. In the case of PNIPAM, phase transition is induced when critical temperature 34°C is achieved. Tsuboi et al. reported laser-induced reversible volume phase transition of PNIPAM in D₂O.⁵⁹ PNIPAM gel is in a swollen hydrate phase below a critical temperature, and in a collapsed dehydrate phase above the temperature. Photon pressure accelerates the dynamic of phase transition, and shift the transition point to lower temperature. At the focal point, where the photon pressure is exerted, dehydration is promoted, based on which they suggest that laser-induced shrinkage. This is quite similar with L-Phe, where phase transition can be regarded as crystal pseudopolymorphism transformation, so we suggest that laser can exclude the heavy water away from the focal spot, leading to anhydrous crystal formation. From another viewpoint, this may be another reason that monohydrate crystals were always generated not at the focal spot.

Under unsaturation (1.7×10^{-2} wt%), there are no L-Phe liquid-like clusters in the initial

solution, but sometimes L-Phe liquid-like clusters are trapped at the focal spot due to concentration fluctuation and laser reveals liquid-like clusters where monomer is changed down the motion at the focal spot and formed local high concentration area (Fig. 3.8, stage (i)). Through the liquid-nucleation like of supersaturation, assembly of L-Phe liquid-like cluster is induced by laser and grew up. It is worth to mention that the size and formation rate of assembly is much smaller and slower than that in supersaturation. It is not large enough to generate monohydrate crystal at 1.7×10^{-2} wt%, so there is no monohydrate crystal formation as shown in Fig. 3.8. On the other hand, anhydrous crystal formation still can be triggered by laser irradiation, where the assembly of large-linked molecules are excluded the heavy water at the focal spot. We conclude that “laser trapping-induced dehydration”

At the middle concentration, liquid-like clusters are less than supersaturation but higher than unsaturation in the initial solution, and the expending rate of assembly of liquid-like clusters are also between 1.7×10^{-2} wt% and 2.9×10^{-2} wt%. Through the same process as shown in Fig. 3.8, monohydrate and anhydrous crystal are generated in the solution, respectively. There is sharp decreasing of probability of anhydrous crystal formation in this concentration range. As we could predict, strong competition between these two crystal formations should happen in this sharp decreasing area. In the case of polarization dependence, there is also the biggest difference between three kinds of polarization and the sharp decreasing at the middle concentration. The detail dynamic and mechanism is discussed as follow.

3.3 Pseudopolymorphism: Laser polarization dependence

Three kinds of laser polarization were performed by quarter wave plate. We examined the probability of anhydrous crystal formation by laser trapping depending laser polarization dependence under various solution concentrations (Fig. 3.9). For convenient and we are more interesting in probability of anhydrous crystal formation, so we reproduced Fig 3.7 to this style.

In this figure, we used the same way to conclude our results, which is based on the first observation of crystal under laser irradiation. Independent on laser polarization, and all the probability of anhydrous crystal formation curves show the similar decreasing trend against the solution concentration. Highest concentration and lowest concentration are promoted the monohydrate and anhydrous crystal formation as we discussed in Fig. 3.7, respectively. At the middle concentration, the most notable difference is performed in three different polarization,

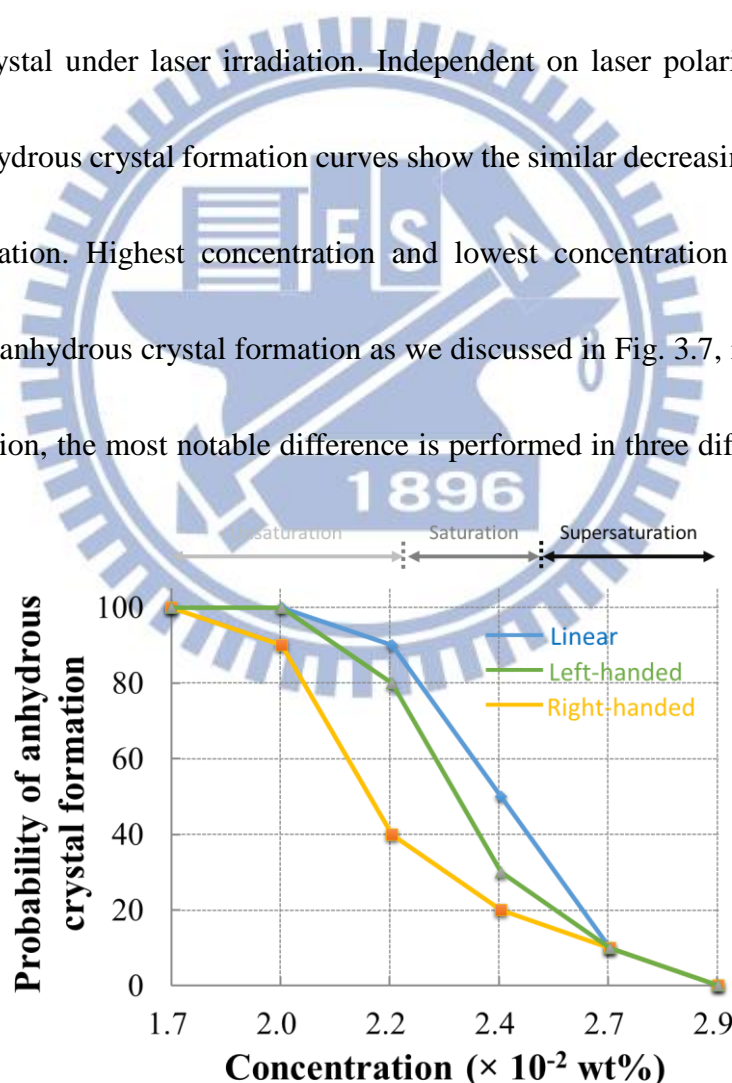


Fig. 3.9 Probability of anhydrous crystal formation under various solution concentration. Blue line; linearly polarized laser, green line; left-handed polarized laser, and gold line; right-handed polarized laser.

and it attracts our attention very much. Therefore, the mechanism and dynamics of laser trapping-induced dehydration and anhydrous crystallization depending on laser polarization at middle concentration is our main discussed target. Yuyama et al. succeed in controlling the crystal polymorph of glycine by intense polarized CW laser beam.⁶⁰ In their study, laser polarization directly affects the molecular alignment, and LP (linearly polarized) laser preferentially generates the γ -form cluster which is LP-liked. That is to say, in order to discuss the crystal polymorphism depended on laser polarization, we should realize L-Phe crystal structure. Unfortunately, single X-ray crystallographic analysis has not been succeeded yet, and only their powder X-ray diffraction study has been carried out.⁶¹ Although X-ray instrumentation and analysis are continuously improving and Korter et al recently proposed a proper molecular geometry configurations using solid-state density functional theory,⁶² but the complete structural determination have not been made yet. Therefore, we here discuss the dynamics and mechanism only on basis of results obtained in this work.

Under linearly polarized laser beam, it shows highest probability of anhydrous formation. It may imply that LP laser traps the large-linked molecules most efficiently. Following this viewpoint, trapping efficiency of large-linked molecules under right-handed polarized laser beam is the worst, and left-handed is between these two. In order to understand more clearly, schematic illustration is shown in Fig. 3.10. In the Fig. 3.10, it shows the mechanism and dynamics under different laser polarization at the middle concentration. Assembly of liquid-

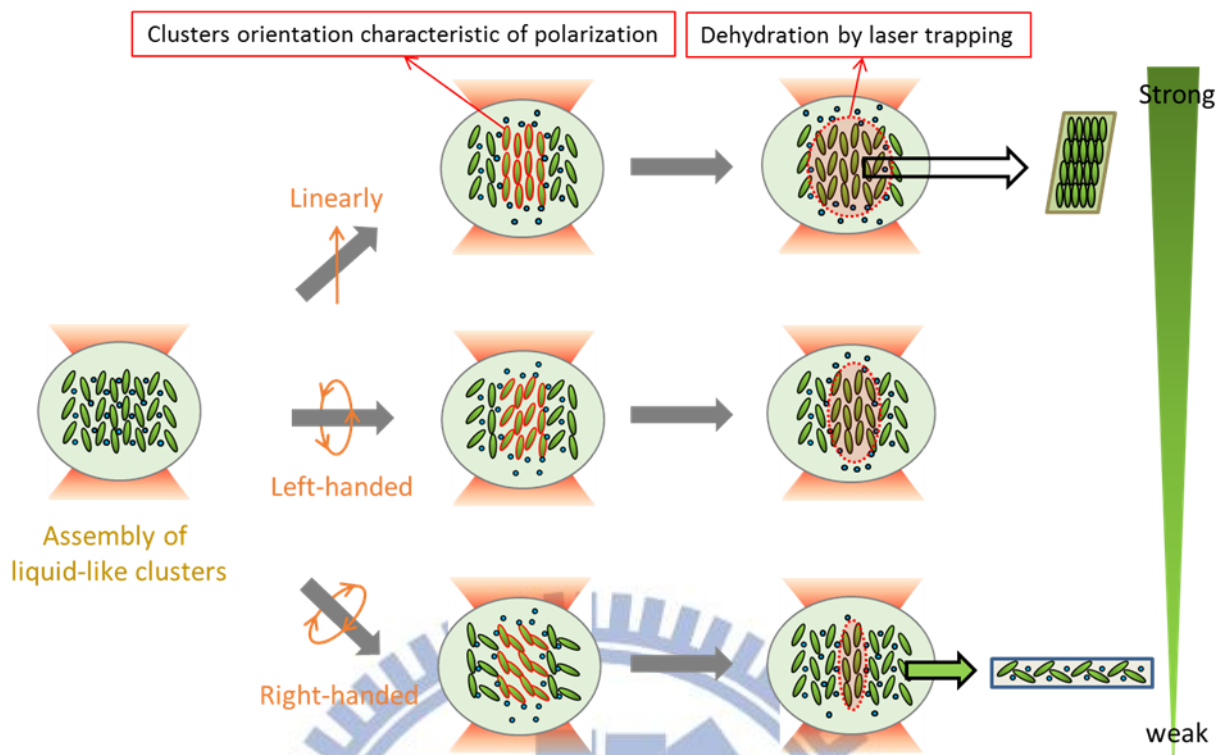


Fig. 3.10 Laser trapping-induced orientation and dehydration in assembly of L-Phe liquid-like clusters under three kinds of laser polarization.

like molecules is induced by efficient trapping, and it grows up by the further irradiation. Firstly, laser pinned orientation is performed in the focal spot. Three kinds of laser polarization generates their own characteristic orientation, and these orientation corresponds to probability of anhydrous crystal formation, which is linear than left-handed than right-handed. It means that linearly orientation is the most close to anhydrous crystal, and right-handed is the most unlike one. Based on the results obtained in this work, I here propose one possibility for this laser polarization-dependent pseudopolymorphism. Laser trapping first increases local concentration, the stable aggregation is formed through the nucleation and the subsequent growth, and eventually the largely linked molecules are prepared in the aggregation. L-Phe is one of chiral organic molecules, so that the formed largely linked molecules also show chirality

independent of the inside molecular structure. The orientation of the largely linked molecules should be influenced by laser polarization because of the chirality. Considering the results in this work, linear-polarized laser beam makes the orientation closest to that of the plate-like anhydrous crystal, while left-handed circularly laser beam makes it least. These results strongly implied that we can control the molecular alignment even crystal polymorphism by laser, which is based on the correlation between laser polarization and molecular chirality. Then, not only laser-pinned orientation but also laser trapping-induced local dehydration which mentioned above triggers the nucleation of anhydrous crystal, leading to crystallization. The size of laser trapping-induced dehydration is depending on the trapping efficiency of large-linked molecules. From our results, LP laser which traps large-linked molecules most efficiently may induced large dehydration easily. On the contrary, left- and right-handed laser is worse than the LP laser due to disliked laser polarization of large-linked molecules. On the other hand, left- and right-handed monohydrate crystal may be observed easily due to small area dehydration compared with LP laser, although laser pinning effect and laser induced dehydration does not allow its formation at the focal spot.

3.4 Summary

We have first succeeded in demonstrating laser trapping-induced dehydration and crystallization of anhydrous crystal of L-Phe in D₂O. The crystallization behavior was much different from that in H₂O, when we explained that the local temperature elevation by laser

irradiation determined the pseudopolymorphism. Actually, crystal pseudopolymorphism in D₂O strongly depends both on concentration and laser polarization. 2.9×10^{-2} wt% and 1.7×10^{-2} wt% are supersaturated and unsaturated solution, and they also indicated the monohydrate and anhydrous crystal formation under laser irradiation, respectively. The mechanism of the pseudopolymorphism against solution concentration under linearly polarized laser irradiation is explained in view of “laser pinning effect” and “laser trapping-induced dehydration.” Laser pinning effect indicates molecular alignment pinned by laser is not suitable for monohydrate crystallization, and it occurs at a point where molecular alignment become disordered away from the focal spot. In other words, anhydrous crystal structure or cluster is LP laser liked which is generated at the focal spot. Then, laser trapping-induced dehydration illustrates the interaction between large-linked molecules is enhanced by laser trapping, and it makes large-linked molecules closet and excludes the heavy water away from the focal spot, leading to anhydrous crystal formation. On the contrary, the dehydration may suppress the hydration at the focal spot. As reported by Yuyama et al., crystal polymorph can be controlled by polarized laser. In our study, it shows the similar result on polarization dependence. Three kinds of polarized laser show the different ability of dehydration which is reflecting the probability of anhydrous crystal formation. For instance, we could use the LP or right hand laser to get the best probability of anhydrous or monohydrate crystal, respectively.

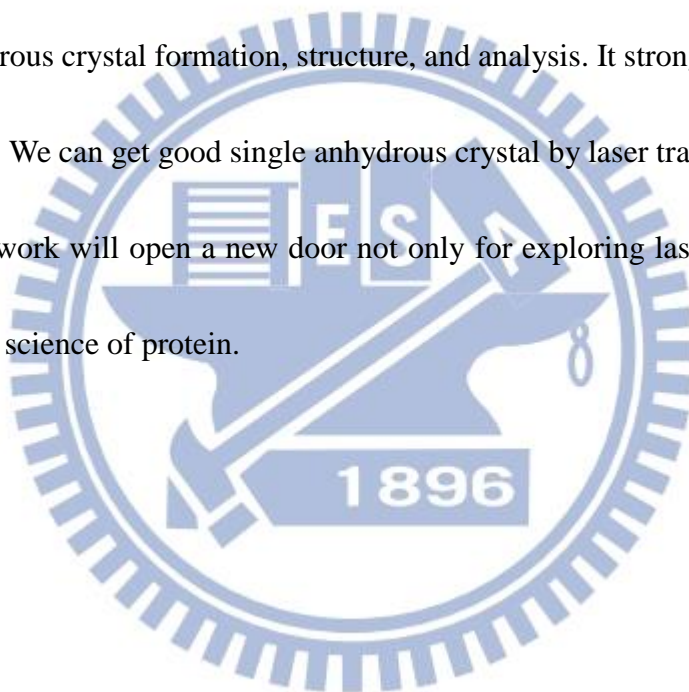
4. Conclusion

In this study, we examined laser trapping crystallization of L-Phe in D₂O and how experiment parameters such as solution concentration, and laser polarization affects the pseudopolymorphism. Firstly, we examined probability of anhydrous crystal formation depending on the initial solution concentration. The laser trapping crystallization provided two different types of the needle- and plate-like crystals and induced either only one of them in most samples. Notably, the anhydrous crystal was always observed at the focal spot, while the monohydrate one was formed at 1 to few micrometer away from the spot, independent of solution concentration. We confirmed that both crystal pseudopolymorphism were generated by focused laser. FT-IR measurement revealed that the needle- and plate-like crystals were ascribed to be monohydrate and anhydrous forms, respectively. We found that independent of laser polarization, solution concentration greatly contributed to change of the probability, and high and low solution concentration accelerates the probability of monohydrate and anhydrous crystal formation, respectively. In particular, the formation probability in middle concentration depends on laser polarization, which controlled dehydration during the crystallization process.

The dynamics and mechanism was discussed in view of formation of cluster assembly characteristic under laser trapping and laser trapping-induced dehydration in the assembly depending on laser polarization. Laser trapping of the liquid-like clusters in solution increased local concentration and eventually small assembly of the clusters was formed through the liquid

nucleation. The needle-like crystallization far away from the focal spot was explained on basis of the assembly characteristic under laser trapping and “laser pinning effect”. Thus, in D₂O solution, the dehydration is not due to laser heating exceeding the transition point, due to laser trapping of the clusters.

Finally, anhydrous crystal formation have not published yet in addition to temperature elevation and our work “laser trapping crystallization.” Furthermore, there was no literature about single anhydrous crystal formation, structure, and analysis. It strongly implies our works are very important. We can get good single anhydrous crystal by laser trapping. These findings mentioned in this work will open a new door not only for exploring laser trapping chemistry but also for crystal science of protein.



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