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Research Paper

Small and Macromolecules Crystallization Induced by Focused Ultrafast Laser

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The structure based drug design has been limited by various factors that include protein crystallization, which is one of the most challenging tasks in this area. It has been proved unequivocally that X-ray crystallography is highly trusted technique for three dimensional structure determination of small- and macro-molecules. It has provided the definite solution for several key areas such as structure based drug design, site directed mutagenesis and elucidation of enzyme mechanisms. In X-ray crystallography, growing good quality of crystals for structure determination is always, the rate limiting step. Various approaches including laser induced crystallization technique has been reported to tackle this issue. We report on the crystallization of small- and macro-molecules under focused optical radiation from a femtosecond laser (λ = 800 nm). The efficacy of this technique is proved by crystallizing different samples such as sodium chloride and urea, and by comparing the results to those crystals obtained by conventional methods. In addition, the unique capability of the developed technique is demonstrated by crystallizing the three chalcone compounds that are difficult to crystallize using conventional methods. Furthermore, the developed technique is extended to crystallize protein molecule (lysozyme) and the results indicate that present technique is a potential alternative tool to crystallize biomolecules.

Key Words: Chalcone Compounds; Femtosecond Laser; Lysozyme; NaCl; Urea; X-ray Crystallography

Introduction

Crystals are ubiquitous in nature and find wide applications, ranging from daily life to advanced scientific research of biomolecules (Blundell, 1996). The three dimensional atomic arrangement of atoms inside a crystal determines the properties of solids and can be investigated using X-rays (Davis *et al*., 2003). The X-ray crystallographic technique helps us to understand biological molecules such as protein or DNA by investigating their crystals (Kindt *et al*., 1991). Even though this leading technique is used for studying the molecular structure of several biological and non-biological molecules and related properties, it has been playing a major role in drug design (Gane and Dean, 2000). But this technique largely depends on the quality of crystals being investigated (Blundell, 1996). Crystallization process involves various steps including sample preparation, purification and characterization (Chayen and Saridakis, 2008). In general, crystallization process is governed by the laws of thermodynamics. Thermodynamically, the proteins (for example lysozyme) as well as small molecules (for example NaCl) crystallize in the same fashion. In both cases, the solution has to be in supersaturated state. In the case of small molecules, the supersaturation level is normally achieved by heating followed by slow cooling at room temperature. At room temperature, the solution will be in metastable state and the nucleation/crystal will appear. In the

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case of proteins, the solubility of the protein not only depends on the temperature but also on various other factors such as concentration, pH, salt present in the buffer etc. These co-factors help in realizing supersaturated state for a protein (Chayen and Saridakis, 2008; Yoshikawa *et al*., 2009). In general, the goal is to grow a few single crystals of suitable size (typically 0.2-0.4 mm) in at least two of the three dimensions for the X-ray diffraction studies rather than obtaining large number of small crystals. In order to obtain a good quality data, crystal size of minimum 0.2 mm x 0.2 mm x 0.2 mm is necessary for small molecules. Though the situation is similar for protein molecules, the advanced synchrotron radiation data collection facilities help to obtain high resolution data for even smaller crystal size (Coulibaly *et al*., 2007; Standfuss *et al*., 2007). Recently Trypanosoma brucei procathepsin B structure has been determined using the X-ray source of PETRA III with the micrometer-sized crystals of an average 9 μm^3 (Gati *et al*., 2014). However obtaining nucleation/suitable good quality crystals still remains challenging. For example, several attempts to crystallize one of the arsenic detoxification proteins, arsenic metallochaperone ArsD, which is responsible for delivering arsenic to its partner protein ArsA with the ligand arsenic as well as with the ArsD-ArsA complex is not successful so far (Abdul Ajees *et al*., 2011). Similarly, crystallization of human arsenic reductase (Cdc25) with arsenic and its binding partners Glutaredoxin (Grx1/Grx2) remains challenging (Bhattacharjee *et al*., 2010). To overcome this difficulty, several methods are proposed. Among these, light induced crystallization is becoming an emerging technique, largely due to its inherent advantages. Several organic, inorganic and protein molecules are crystallized using various light sources (Hosokawa *et al*., 2005; Yoshikawa *et al*., 2009; Yennawar *et al*., 2011; Basu *et al*., 2012; Yoshikawa *et al*., 2014). In this work, we report crystallization of small- and macro-molecules by a focused femtosecond laser (λ = 800 nm). We have investigated the crystallization behaviour of seven small molecules (NaCl, urea and five chalcone compounds) and a protein (lysozyme). The efficacy of this technique is demonstrated by crystallizing three chalcone compounds (out of above-mentioned five) that were difficult to crystallize by conventional methods.

Materials and Methods

Sample Preparation

The analytical or reagent grade chemicals and other components were purchased from Sigma-Aldrich (St. Louis, MO), Merck (New York, United States.), SRL (Mumbai, Maharashtra) and LOBA (Tarapur, Maharashtra). NaCl (5M and 7M) and urea (2M) were prepared in water and other organic compounds were prepared using methanol. Lysozyme solution (50 mg/ml) was prepared in 100 mM sodium acetate (pH 4.5). In all the experiments, conventional crystallization methods were used as control. In case of NaCl and urea, 20 µl volume of sample were used in a cover slip. For chalcone compounds, one ml of sample in 5 ml glass beakers was used. The hanging drop method using Linbro 24 well plates (HR3-110; Hampton Research, Aliso Viejo, CA) was employed for crystallizing lysozyme protein.

Experimental Setup

A schematic representation of the experimental setup is shown in Fig. 1 (Thomas *et al*., 2013). In brief, a femtosecond laser (Femtolaser scientific XL200, Femtolasers, Austria) operating at 800 nm with a pulse duration of 60fs and repetition rate 5.2 MHz is used as a source. The output energy of the laser is controlled to experimentally required power level using a half-wave plate and thin film polarizer and laser beam is focused inside the solution via an objective (10X, 0.25 NA for protein, 4X, 0.10 NA for small

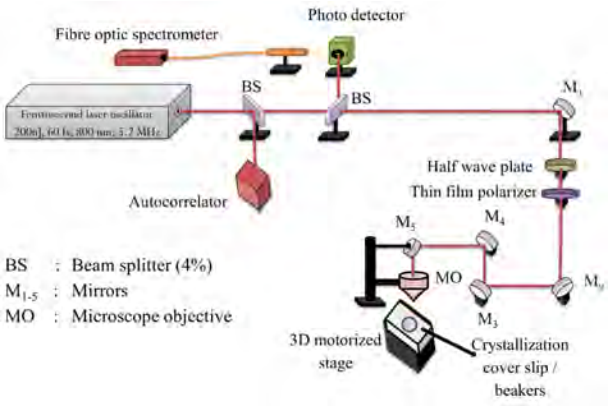


Fig. 1: Schematic representation of femtosecond laser based crystallization set up

molecules) coupled to an upright microscope with attached camera (Nikon Eclipse 80i). The small molecules in 1ml volume of the solution are exposed for 5 minutes whereas 5µl volume of lysozyme protein mixed with equal volume of well buffer sample are exposed for varying time scale of 1, 2, 3, 4 and 5 minutes. Immediately after the laser exposure, all samples were incubated at 292 K.

Results and Discussion

Optimization

Initially, the experimental setup was optimized by investigating the crystallization behaviour of two well-known samples NaCl and urea under femtosecond laser irradiation (average power 600mW; energy fluence 970 mJ/cm^2). For these measurements, NaCl solution (5M and 7M) and urea (2 M) were used as starting materials and corresponding concentration of the particular samples under non-illuminated condition was used as the control. The time taken for crystallization of the samples was measured and the results are shown in Table 1. From Table 1, it is clear that focused femtosecond laser irradiation reduces the crystallization time by a factor of 10 for NaCl and by a factor of 2 for urea compared to non-irradiated samples. These studies unequivocally manifest femtosecond laser irradiation can accelerate the crystallization process in a significant manner.

Crystallization of Chalcone Compounds

The above-mentioned method is extended for five different chalcone compounds. In the case of chalcone molecules, 1 ml of supersaturated solution in a base solvent (methanol) is taken in a 5 ml glass container and exposed to femtosecond laser radiation (300 mW; energy fluence 70 mJ/cm^2) for 5 minutes. Following the exposure to laser radiation, the container is covered with paraffin film with small holes. The solution is then allowed for slow evaporation and the process of crystal growth is monitored every 24 hours using a microscope. It is observed that the high quality crystals that are suitable for X-ray diffraction studies are obtained in a time span of 3 to 4 days. Our experimental observations are summarized in Table 2. In the present study, we crystallized five different chalcone compounds with size ranges from 0.1 mm to 0.5 mm using femtosecond laser. Reports

Table 1: Optimization results for NaCl and urea

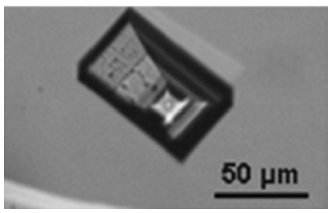
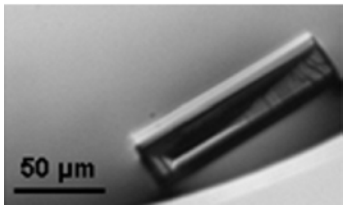
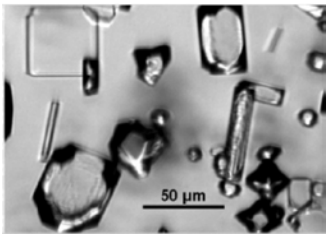
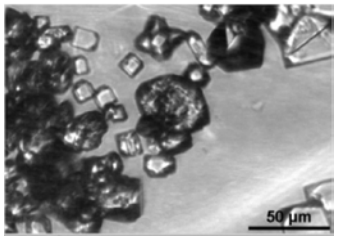
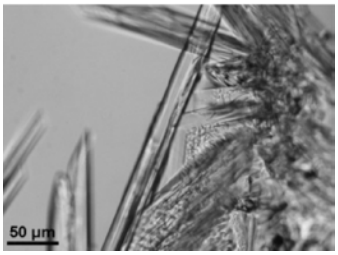
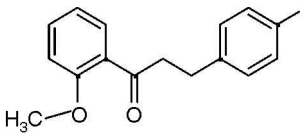
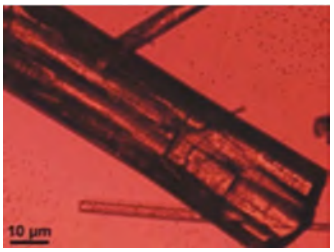
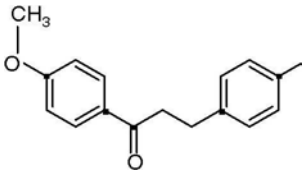
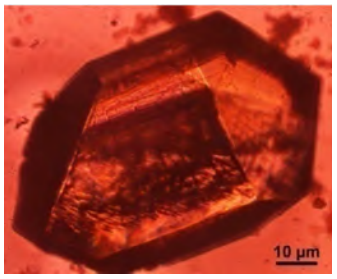
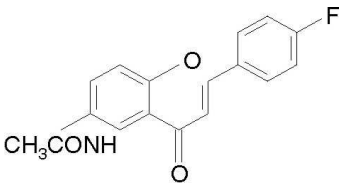
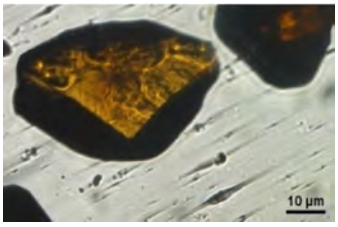
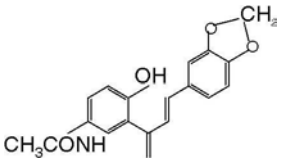
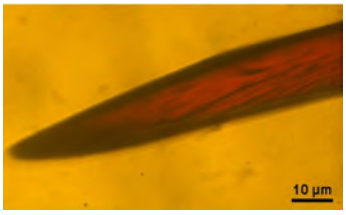
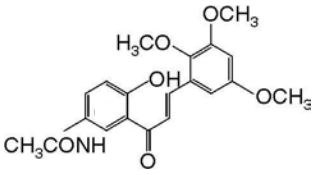
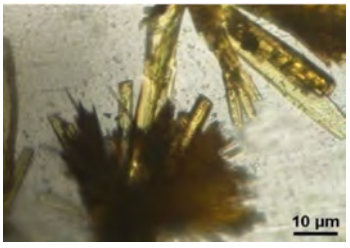
Compound	5 M NaCl	7 M NaCl	2 M Urea
Control			
Time taken for crystallization	20 minutes	8 minutes	23 minutes
Under Laser			
Time taken for crystallization	2 minutes	58 seconds	10 minutes

Table 2: Crystallization results of chlacone compounds

S.No.	Chemical name	Chemical structure	Crystal images
1	3-(4-hydroxyphenyl)-1-(2-methoxyphenyl) propan-1-one		
2	3-(4-hydroxyphenyl)-1-(4-methoxy phenyl) propan-1-one		
3	1-(5-acetamido-2-hydroxy-4-fluoro phenyl)-3-phenyl propen-2-one		
4	1-(5-acetamido-2-hydroxy phenyl)-3-(2,3,5-trimethoxy phenyl)- propen-2-one		
5	1-(5-acetamido-2-hydroxy phenyl)-3-(3,4-methoxylene dioxy phenyl) - propen-2-one		

discussing about the crystallization of the first two compounds (Table 2) using techniques other than laser-based crystallization technique have already appeared (Shubalaxmi *et al.*, 2013). More importantly, we are successful in obtaining good quality crystals from other three samples also, which are very difficult to crystallize through conventional methods.

X-ray Crystallographic Analysis of Chalcone Compound

A single crystal X-ray diffraction experiment was carried out to understand the capability of laser to

induce crystallization. The preliminary cell determination data collection for one of the chalcone compounds, 3-(4-hydroxyphenyl)-1-(4-methoxy-phenyl) propan-1-one, crystallized under conventional methods and under femtosecond laser source was collected using Bruker SMART APEXIII CCD diffractometer with Mo $K\alpha$ radiation under temperature 293 K (Bruker 2008). Both cell parameters are refined and evaluation of crystal packing parameters indicates that the crystals belong to monoclinic system. The cell dimension for the crystals grown under conventional method is a = 10.72 Å; b = 8.22 Å; c = 16.46 Å; α = 90 °; β = 99.33°, γ = 90° and for the crystals grown under femtosecond system is a = 10.34 Å; b = 7.97 Å; c = 16.06 Å; α = 90°; β = 99.22°, γ = 90°. The diffraction experiment results suggest that (1) the crystals grown under ultrafast laser can yield a good diffraction quality data, (2) the laser has not changed the crystal packing and had assisted in speeding up nucleation/crystallization.

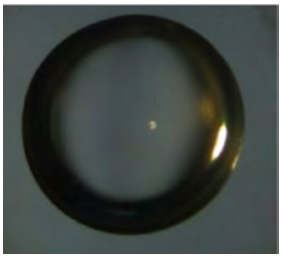
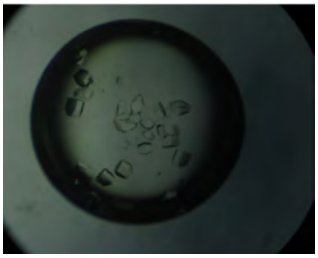
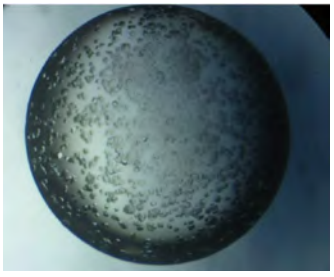
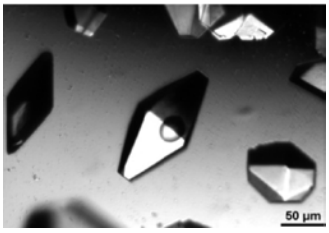
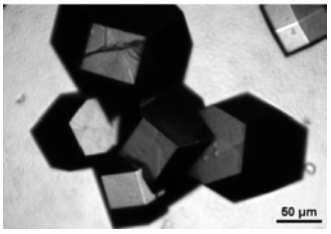
Crystallization of Lysozyme

Crystallization of lysozyme protein was carried out under the femtosecond laser system and non-irradiated sample was kept as control for monitoring. The crystallization experiments were performed using well-known hanging drop method (Chayen and Saridakis, 2008). The reservoirs of the well plates were filled with 1ml of the precipitant solution containing 100 mM sodium acetate (pH 4.5) and 1M NaCl buffer. The 10 µl drops were prepared by mixing equal volumes of the protein sample and reservoir solution. The drops were then subjected to femtosecond laser (100 mW power; energy fluence 160 mJ/cm^2) for varying times of 1, 2, 3, 4 and 5 minutes. After the irradiation, the mixture was monitored under the microscope to avoid false positive identification of protein crystals. The crystallization plate was kept under temperature of 292 K in clean environment without vibration. The plate was checked every six hours for first 24 hours, and then every few days. Up to 18 hours, no nucleation/crystal formation was observed in control, whereas, good quality lysozyme protein crystals appeared in all femtosecond laser irradiated samples. The result of this experiment is summarized in Table 3.

Possible Mechanism of Nucleation Under Laser Irradiation

To understand the possible nucleation mechanism, we

Table 3: Lysozyme (50 mg/ml) crystals obtained after 18 hours

	Control	Under Laser	Under Laser
Laser exposure time	0 minute	1 minute	5 minutes
Lysozyme			
Magnified images			

have recorded the UV-Vis-NIR absorption spectrum (200 nm to 900 nm) of all the samples investigated here using a commercially available Spectrophotometer (V-650, Jasco, U. K). The study carried out for various concentrations of sodium chloride, sodium acetate and urea samples do not show any appreciable absorption in the studied spectral range (data not shown). However, chalcone compounds showed absorption in the UV range (300-400 nm), while lysozyme protein dissolved in 0.1 M sodium acetate and 1 M NaCl showed strong absorption around 280 nm that arise from amino acids such as tryptophan, tyrosine and cysteine. The chalcone compounds showed absorption around 340 nm, which is a known characteristic of these compounds (Song *et al*., 2003). On the other hand, we have observed the gaseous bubble formations around the focal point of samples. Although samples such as NaCl, urea and lysozyme may not absorb the 800 nm light, the intensity of femtosecond laser radiation at the focal point is high enough to induce higher-order multiphoton absorption that lead to the shock waves and cavitation bubble generation. The perturbation arising from these phenomena in the surrounding media near the focal point of the laser beam can trigger the nucleation. The present experimental observations are consistent with earlier studies in similar direction (Yoshikawa *et al*., 2006; Murai *et al*., 2011).

Conclusion

In this report, we have demonstrated the efficacy of the femtosecond laser source to grow suitable crystals for applications such as X-ray crystallography. The results suggest that femtosecond laser system has an advantage of obtaining good quality crystals as compared to crystals obtained from conventional methods. Further, an optimization of different parameters of crystallization as well as experimental set up will be carried out to crystallize various proteins, which are difficult to crystallize for example protein-protein complexes, protein-nucleic acid complexes, viruses, immune complexes and membrane proteins.

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