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REVIEW PAPER

A new strategy for protein crystallization : Effect of ionic liquids on lysozyme crystallization and morphology

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Abstract−Protein crystallization is a complex physical and chemical process. The high-quality protein crystal is still a persistent bottleneck to the application of X-ray crystallography in structural biology. The additives may promote formation of crystal nucleus and subsequent growth in protein crystallization. As a distinct material, ionic liquids (ILs) have aroused great attention and interest for protein crystallization due to their unique properties. We reviewed the progress of protein crystallization and reported research about protein crystal morphology control by ILs, as crystal growth template, in aqueous solutions. ILs encourage changes in some cases in terms of growth morphology and crystal size. The effect of ILs on lysozyme growth morphology can be attributed to changing interaction among lysozyme molecules in aqueous solutions. This work can provide some initial insight into the preparation of high quality crystal and the development of new crystal form.

Keywords: Protein Crystallization, Crystal Morphology, Ionic Liquids, Lysozyme

IMPORTANCE TO PROTEIN CRYSTALLIZATION

The knowledge of the three-dimensional structure of biomolecules could provide useful information about the mechanism of molecular behavior at the atomic level. Such information forms a very important part of biology, as well as medicine, biotechnology, and any field dependent upon new applications of biomacromolecules [1,2]. X-ray diffraction, which is the most reliable method to determine the structure of biomacromolecules, plays a fundamental role in connecting the dots between genomic data and biological function by providing accurate structural information to resolve several significant research problems [3,4]. Solving protein structures by X-ray crystallography is contingent upon the availability of ordered, high-quality macromolecular crystals. Therefore, obtaining good quality protein crystals is a critical step within the process of X-ray structural analysis.

CURRENT SITUATION AND PROGRESS IN PROTEIN CRYSTALLIZATION

Up to the present, preparation of high-quality macromolecular protein crystals, in particular ones that have low solubility and easily aggregate, and even a large percent of soluble proteins, is still a persistent bottleneck to the greater application of X-ray crystallography in structural biology [3,5]. Although many techniques for protein crystallization, including high-throughput cloning [6], protein production [6,7], purification [8], automated crystallization screening [9], application of nanotechnology [10] to the field introduced microfluidic devices and biocompatible materials with new properties, have been developed, successful crystallization is still largely empirical and operator-dependent. It can be concluded that there is no single universal method for the crystallization of protein. However, inspection of the Protein Data Bank (PDB) [11], with its impressive num-

Table 1. Dominant themes and new trends of biological macromolecules in ICCBMs [7]

Dominant themes	New trends highlighted
Mechanisms and physics of protein crystal growth, particular attention to macromolecular solution properties and the effects of ions, solvents, and cosolvents	Novel crystallization methods as exemplified by crystallization in gels, under oil, under magnetic field, under high pressure, under an external electric field
Purity, and related to it, impurities in protein crystallization	Nucleation initiation on mica plates, and by stirring
Crystal perfection and defect structure, Comparisons with the solution growth of small molecules	Novel crystallization strategies based on chemical considerations: combinatorial approaches, rational use of additives
Physical processes related to microgravity	

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ber of structures, would seem to indicate that great advances have been made in protein crystallization. Scientific results reported at the International Conferences on the Crystallization of Biological Macromolecules (ICCBMs) have contributed substantially to these advances. Crystallogenesis research trends in the last two decades as seen from the International Conferences on the Crystallization of Biological Macromolecules (ICCBMs) are listed in Table 1.

As already described [8,12,13], many methods of protein crystallization exist. However, insight into the nucleation and crystallization processes is still a challenge [14]. The concept of nucleation and its connection to crystal growth has to be understood in order to select the appropriate crystallization method [15]. The analysis of nucleation applied to protein crystal growth, protein solubility and temperature dependency has proven to be useful when growing crystals suitable for X-ray analysis [16,17], which can give rise to valuable information about the supersaturation conditions as well as on crystal quality improvement. The mechanisms of crystal growth are also particularly important for understanding the history of the crystallization process. Scanning electron microscopy, as well as atomic force microscopy, have pioneered efforts in the investigation of crystal growth mechanisms [18,19]. Some different experimental techniques for the measurement of nucleation and crystal growth kinetics were published [20,21]. Despite all this, additional research is needed to explore physical and chemical properties of macromolecular solutions and the change of protein-protein interactions.

THE FACTORS INFLUENCING PROTEIN CRYSTALLIZATION

Except for factors of protein itself, such as protein purity, aggregation state, origin, hydrolysis, symmetry, stability and isoelectric point, factors impacting protein crystallization can be divided into two categories: physical and chemical aspects.

1. Physical Aspect

With regard to macromolecular physic factor, including temperature, gravity, pressure, magnetic field, crystal defects and so on, the relative importance of physical factors affecting the crystallization process is not fully understood. However, inspection of the Protein Data Bank, with its impressive number of structures, seems to indicate that the great advances have been obtained in protein crystallization. This is true to some extent and is due to advances in physical aspects such as sample preparation, screening methods, and automated crystallization technologies. A better understanding of the protein crystal growth has also contributed to more rational approaches to protein crystallization. Scientific results reported at previous ICCBMs have contributed substantially to these advances [22]. Waizumi indicated a novel pH-dependent nucleation and growth of insulin crystals via liquid droplets, where the viscosity of the crystallizing samples also plays a critical role [23]. Koizumi, et al., exploring the mechanical properties of lysozyme crystals, showed their connection to elasticity and characterized crystal imperfections [24]. Particularly impressive were the results of two Japanese scientists from Tohoku University at Sendai. They presented new instruments and a host of intriguing observations on molecular diffusion on crystal surfaces by advanced confocal microscopy and exquisitely sensitive interferometric approaches to analyze growth step advancement and growth mechanisms [25,26]. Bergfors reviewed fundamental approaches and their practical application, whereas Chayen described the use of a new substrate for the promotion of crystal nucleation [22].

2. Chemical Aspect

Chemical factors influencing the crystal growth of protein include pH value, precipitant types and solution concentration, specific ions, ionic strength, supersaturation degree and the existence of impurities, in which the impact of the precipitants and additives on protein crystallization has received considerable attention.

For protein crystallization, the main impact of precipitant is on the solvent (water) rather than the protein molecules. Two types of protein precipitant, salts and organic solvents, are based on a completely different mechanism. Salt can damage the hydration layer of the protein surface, reduce binding capacity of the protein and water, and increase the binging capacity between proteins. Organic precipitation agent can reduce the dielectric constant to weaken the electrostatic repulsion and polarity. However, the purpose of the various precipitation agents is actually to increase the attraction power among the proteins and promote the generation of chemical bonds which constitute the protein crystals.

3. What Additives Can Assist

The additives, as an important factor, on influence of the protein crystallization may increase the protein solubility, reduce the surface energy of the crystal, and promote formation of crystal nucleus in protein crystallization [27]. The additives have many different types, and there is no fixed form. A large number of works have indicated that the control of protein crystallization by additives is potentially very useful for crystal structure determination, as well as for industrial applications [28]. Additives are normally applied to improve the order of poorly diffracting crystals or to reduce the branching of crystals and the growth of crystal clusters through suppressing random aggregation of protein molecules [29,30]. Some studies have shown that the effects of additives on the solubility of proteins are related to the molecular interactions [2]. To address bottleneck of protein crystallization and improve the crystallization potential of protein, robust crystallization strategies need to be developed. One potential approach is to find new materials or additives specifically recognizing the protein so that interaction in solution is formed that is better suitable for protein crystallization.

4. Uses of ILs in Protein Crystallization

The ILs, as distinct materials, have attracted more and more attention due to their unique physical and chemical properties with low melting points that can exhibit intrinsically useful characteristics such as a wide liquid range, a negligible vapor pressure and a high electric conductivity [31-33]. ILs are considered as the alternative of volatile organic solvents in chemical processing and extraction and have numerous potential applications in many other fields. These compounds have been applied as solvents for organic reactions [34] and for liquid-liquid extraction [35].

One advantage to the use of ILs is the wide range of possible structures. According to types and characters of proteins, ionic liquids can be designed for the crystallization of proteins to optimize and control the crystallization process to meet specific application requirements. The applications of ILs related to the protein crystallization are being reported because of the potential for productive interaction between ILs and protein [36,37]. Additionally, ILs are useful for improving the monodispersity of proteins which exhibit multiple aggregation states. ILs as additives have been applied into protein

crystallization [37,38]. The results indicated that ILs are favorable for macromolecule crystallization and good diffraction crystals were obtained. The kinetics of protein crystallization was significantly enhanced by addition of water-soluble ILs. Even at higher salt concentrations, precipitation could be avoided reliably. The crystal polymorphism could be reduced compared with experiments without ILs. As a result, the addition of ILs tends to result in larger crystals. In carrying out this function, the additive presumably affects specific intermolecular interactions so that more defined and a more ordered or higher dimensional solid state is attained.

Judge et al. [37] used sixteen ILs for the crystallization behavior of five model proteins. The results illustrated that the proteins produced changes in crystal morphology and significantly increases in crystal size in some cases by adding ILs. Crystals grown using ILs as additives provided X-ray diffraction resolution similar to or better than that obtained without ILs. At the same time, ILs were used as additives for the crystallization of the poorly diffracting monoclonal antibody. The ILs, triisobutyl-(methyl)-phosphonium-p-toluenesulfonate and 1-butyl-3-methylimidazolium tetrafluoroborateraflu-oroborate, improved the crystallization behavior and provided improved diffraction, resulting in the determination of the structure, in contrast to the use of additives from a commercially available additive screen.

Lange et al. [39] suggest that ILs can be excluded from the protein surface to stabilize proteins and promote salting out in working, while additives that preferentially bind favor protein denaturation and solubility. The results of some researches also confirmed that the same space group was found as compared with protein grown in the solution without the IL and no IL ions in the crystal structure. The possible effect of ILs on crystal morphology and crystal size was likely due to subtle changes in solution conditions providing a change in protein solubility or crystal nucleation and growth kinetics, rather than as a result of protein binding. Therefore, how the ILs affect the crystallization process does not indicate a single, clear mechanism. However, it is noted that ILs have the potential to improve macromolecules crystal by a screen of ILs and control of crystallization conditions.

A TYPICAL CASE: ILS' EFFECT ON LYSOZYME CRYSTALLIZATION AND MORPHOLOGY

1. The Effect of ILs on the Interactions Between Protein Molecules

Imidazolium-based ILs have also been used to enhance protein folding and suppress aggregation [39]. Given these applications with biomaterials and their ability to participate in ionic, hydrophobic, and hydrogen bond interactions, ILs are potential additives for use in protein crystallization. Some probable mechanisms of the effect of ILs on protein crystallization were proposed that ILs can influence the interactions between protein molecules, alter surface energy of the crystal such that those formed are more defined and a more ordered or higher dimensional solid state is attained [2,37]. Some works [37,39] have already indicated that the effect of additives on macromolecules solubility strongly correlates to the strength of molecule interactions. In our previous work [40], the ILs 1-butyl-3-methylimidazolium tetrafluoroborate ([C4mim]BF_4), 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl), 1-butyl-3-methylimidazolium bromide ([C_4mim]Br), and 1,3-dimethylimidazolium iodine ([dmim]I) were employed to investigate their effects on the solubility of lysozyme in aqueous solutions at pH 4.5. The results show that lysozyme solubility increases with the addition concentration of [C_4mim]BF_4 and [C_4mimCl] and is nearly invariable with the increase of [C_4mim]Br concentration and slowly decreases with the increase of [dmim]I concentration. The increase of lysozyme solubility with [C_4mim]BF_4 and [C_4mim]Cl with concentration raising demonstrates that either repulsive interactions are induced or attractive interactions are reduced among lysozyme molecules. The solubility decrease of lysozyme after adding [dmim]I shows that either attractive interactions are enhanced or hydrophobic sites with a salt are promoted to form, while with the presence of [C_4mim]Br, the effect of [C_4mim]Br on lysozyme solubility is negligible, indicating the minimum effect on molecules interactions is introduced.

2. The Effect of ILs on Nucleation

The first crystallographic bottleneck in structural determination of protein is likely to the ability to nucleate and subsequently grow a protein crystal suitable for X-ray diffraction. In our work [41], the effect of two imidazolium-based ILs, [C_4mim]Cl and [dmim]I, on the nucleation kinetics of lysozyme was investigated by determining the nucleation induction time and evaluating nucleation parameters. Compared with solutions without IL addition, the critical free energy change, size, and molecular number of critical nuclei decreased and the nucleation rate increased after the addition of [C_4mim]Cl. It is indicated that the addition of [C_4mim]Cl reduces attractive interactions among the lysozyme molecules. In contrast, the critical free energy change, size, and molecular number of critical nuclei increased and the nucleation rate decreased after the addition of [dmim]I. It is supposed that the addition of [dmim]I enhances attractive interactions among the lysozyme molecules and the promotion of the formation of hydrophobic sites with salt.

3. The Effect of ILs on Crystal Growth Morphology

As crystallization additives, the selected ILs influence crystallization behavior of lysozyme by providing larger crystals or generating different crystal morphologies. As shown in Fig. 1, the polarizing microscope was used to analyze crystal morphology in the presence of ILs. Compared with no ILs (Fig. 1(a)), the morphology and size of lysozyme crystals were significantly improved. A plate-like crystal morphology of lysozyme in the presence of [C_4mim]BF_4, [C_4mim]Cl and [C_4mim]Br was observed, corresponding to Fig. 1(b), 1(c) and 1(d), respectively. The crystal morphology of lysozyme in the presence of [C_4mim]Cl was the prettiest. As for adding [dmim]I, it is noted that the case is completely different from adding [C_4mim]BF_4, [C_4mim]Cl and [C_4mim]Br in crystal morphology as shown Fig. 1(e). The needle-like lysozyme crystals in morphology are observed. The difference in between cations [C_4mim] and [dmim] in aqueous solution might be responsible for the shape transition of lysozyme crystal from plate to needle.

There is the possibility that ILs concentration affects the strength of specific intermolecular interactions among proteins or IL and individual protein, and this influence will likely be variable with concentration change. Changes in crystal morphology and size for lysozyme were shown in Figs. 2 and 3.

3-1. Crystals Formed from [C_4mim]Cl

The influence of [C_4mim]Cl addition concentration on crystal morphology of lysozyme is shown in Fig. 2. Crystals present a prism

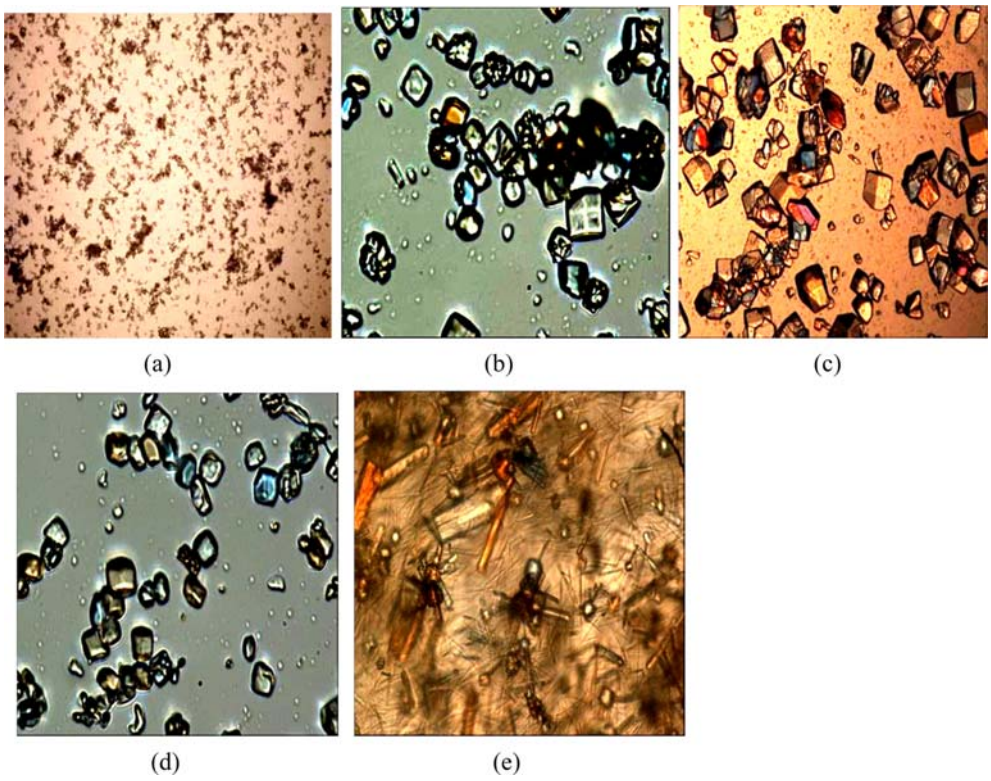

Fig. 1. Polarizing micrographs of lysozyme crystals obtained with 1% addition concentration of ILs in buffer at pH 4.5: (a) no additive; (b) [C_4mim]BF_4; (c) [C_4mim]Cl; (d) [C_4mim]Br; (e) [dmim]I.

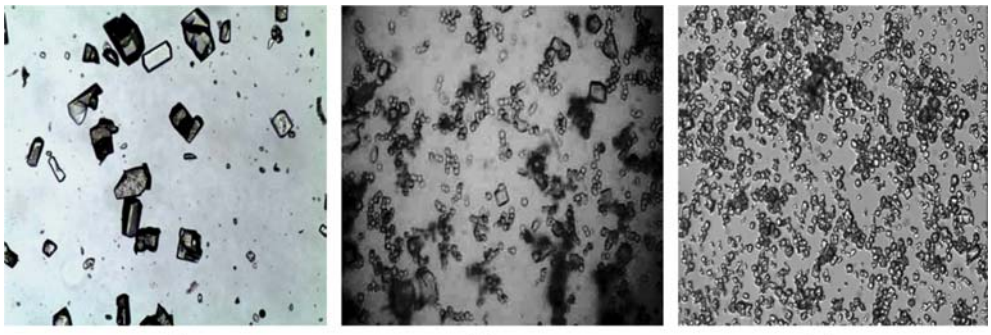

Fig. 2. Micrographs of lysozyme crystals obtained with additive in 0.10 M NaAc/HAc buffer at pH 4.5: (a) 1% [C_4mim]Cl; (b) 3% [C_4mim]Cl; (c) 5% [C_4mim]Cl.

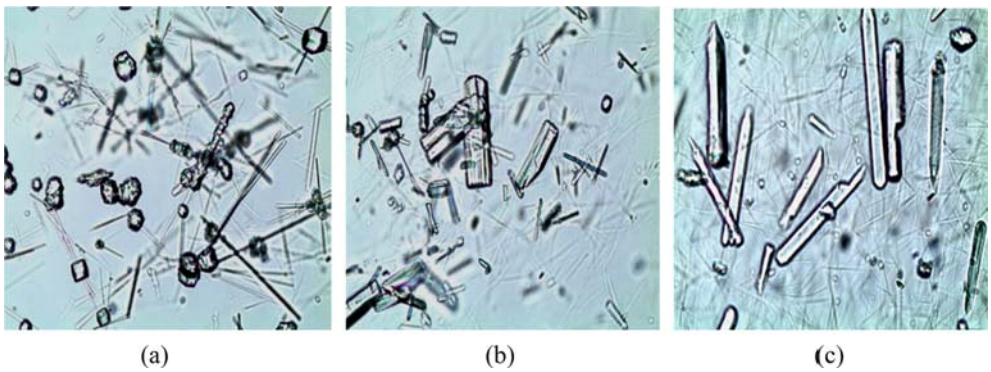

Fig. 3. Micrographs of lysozyme crystals obtained with additive in 0.10 M NaAc/HAc buffer at pH 4.5: (a) 1% [dmim]I; (b) 3% [dmim]I; (c) 5% [dmim]I.

in morphology and the crystals are clear and regular under 1% addition concentration and the crystal size becomes smaller with increasing [C_4mim]Cl addition concentration.

3-2. Crystals Formed from [dmim]I

As for adding [dmim]I, it is noticeable that the crystal morphology is completely different in this case as shown in Fig. 3. The lysozyme crystal morphology changes from plate-like and needle-like mixture to purely needle-like crystals, suggesting a phase transition may occur. With regard to adding 1% [dmim]I, lysozyme crystals appear needle-like and plate-like. However, when 3% [dmim]I was added, crystal morphology became needle-like without plates, while when 5% [dmim]I was added, the thinner needles were formed compared with the case of adding 3%.

CONCLUSIONS

It is a fact that there is no a single universal method for the crystallization of protein, so this focused finding maybe provides an encouragement to search for new possibilities to achieve a proper crystal quality, moving the reader from the classic methods to new strategies.

Limited numbers of ILs have been chosen to investigate how ILs affect lysozyme crystallization behavior in this paper. The ILs produced changes in lysozyme crystal morphology, size and crystallization behavior in some cases. Some probable mechanisms of the effect of ILs on lysozyme crystallization are proposed that ILs can influence the interactions between lysozyme molecules, change solubility, shift thermodynamic equilibrium, alter surface energy of the crystal, and thus affect nucleation in crystallization. Those findings can contribute to a better understanding of the effect of ILs involved in protein crystallization and provide an insight into macromolecular morphology control.

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