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Enhancement of Lysozyme Crystallization Using DNA as a Polymeric Additive

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Abstract: This study reports the first experimental evidence of using DNA as a polymeric additive to enhance protein crystallization. Using three kinds of DNA with different molecular weights—calf DNA, salmon DNA, and herring DNA—this study showed an improvement in the success rate of lysozyme crystallization, as compared to control experiments, especially at low lysozyme concentration. The improvement of crystallization is particularly significant in the presence of calf DNA with the highest molecular weight. Calf DNA also speeds up the induction time of lysozyme crystallization and increases the number of crystals per drop. We hypothesized the effect of DNA on protein crystallization may be due to the combination of excluded volume effect, change of water's surface tension, and the water competition effect. This work confirms predications of the potential use of DNA as a polymeric additive to enhance protein crystallization, potentially applied to systems with limited protein available or difficult to crystallize.

Keywords: lysozyme crystallization; DNA; additive; volume exclusion

1. Introduction

Deoxyribonucleic acid (DNA) is a molecule that consists of nitrogen-containing nucleobases and a sugar phosphate. Highly monodispersed and uniform DNA has been studied for a long period of time as the code of life until early 1980s, Professor Seeman first proposed that DNA could be treated as a polymer [1–3]. Since then, DNA has been utilized as chemical materials and scientists have been trying to endue DNA more new properties by modifying DNA with a variety of functional molecules [4]. The DNA molecule has extraordinary characteristics which include precise recognition and the DNA sequences can be easily programed and designed into different kinds of nanostructures such as DNA origami [5–13]. We have already found that DNA origami can be applied to enhance protein crystallization [14]. This precisely designable and programmable nanostructure is an effective material in the field of protein crystallization. Due to the unique structure of DNA, it has attracted extensive attention and shown great promise for various applications in many fields such as material science and biomedical and pharmaceutical fields [15–17]. However, intrinsic polymeric property has been neglected in protein crystallization.

Protein crystallization is used to precisely define the atomic structure and enable the development of many advances based on the understanding of biological systems such as drug design [18–21]. Many studies have sought for the enhancement of protein crystallization by adding different materials and additives. Traditionally, horse tail hairs and minerals have been added until recent studies discovered the potential use of three-dimensional (3D) nanotemplates, molecular imprinted polymer, porous materials like silicon, and bio-glass in the field of protein crystallization [22–31]. We have previously reported that DNA origami is effective as a seed to promote protein crystallization due to its integral nanostructure [14]. However, the function of DNA as a long chain polymer is not discovered and studied in the field of protein crystallization. The DNA as a long chain polymer is expected to have the following properties: (i) DNA length and sequence can be precisely controlled and designed; and (ii) DNA can be modified with functional groups to have specific interactions with a wide range of biomolecules including proteins, antibodies, and peptides.

We hypothesize that DNA in the solution will cause an excluded volume effect, change of water's surface tension, and water competition with protein molecules. The combination of these effects may create a positive effect for protein crystallization. Therefore, we proposed that DNA can serve as a polymeric material to promote protein crystallization. It is still an unexplored aspect as to whether and how such DNA affects protein crystallization. To answer this question, three kinds of DNA—DNA from calf thymus (calf DNA), DNA from salmon testes (salmon DNA), and DNA from herring testes (herring DNA)—were chosen in our study. Lysozyme was chosen as model protein in this study due to its accessibility and it is widely used in the previous studies [32–34]. Findings from this work would allow the development of new systematic methods for crystallization of biological macromolecules.

2. Materials and Methods

2.1. Materials

Lysozyme from chicken egg white as lyophilized powders, calf DNA (~15,000 base pairs), salmon DNA (~1,000 base pairs), herring DNA (~100 base pairs), sodium chloride (NaCl), and anhydrous sodium acetate (NaAc) were purchased from Sigma-Aldrich and utilized without further purification. Acetic acid (CH_3COOH) was purchased from Guangfu (Tianjing, China). All of these reagents were of analytical grade or above.

2.2. Protein Samples Preparation

Lysozyme solution: lysozyme was dissolved in 0.05 M NaAc (pH 4.6) buffer solution. Protein samples and all of the solution used for crystallization were filtered through 0.22 μm mesh size filters before setting up trials for crystallization. The concentrations of lysozyme were 2.5, 5.0, 7.5, and 10 mg/mL.

2.3. Agarose Gel Electrophoresis

The number of base pairs (bp) for salmon and herring DNA was determined by 1% agarose gel electrophoresis and visualized under UV light. The results obtained for calf, salmon and herring DNA were ~15,000 bp, ~1000 bp, and ~100 bp, respectively (Figure S1).

2.4. Protein Crystallization Experiment

Protein crystallization was performed with 24-well plates using conventional hanging-drop vapor-diffusion method. Crystallization drops were made by mixing 1.5 μL of lysozyme solution and an equal volume of precipitant containing different DNAs: calf DNA, salmon DNA, herring DNA, and blank (without DNA). The drops were equilibrated against 300 μL of precipitant solution in the reservoir well. The precipitant solution used for lysozyme contained 1.0 M NaCl and 0.05 M NaAc (pH 4.6). The concentrations of salmon and herring DNA were: 2.5, 5.0, 10, 15, and 20 mg/mL. Due to low solubility of calf DNA in precipitant solution, lower concentrations of calf DNA were used: 0.1,

0.5, 1.0, 2.5, and 5.0 mg/mL. After the crystallization plates were sealed, the plates were incubated at 4 °C for one week and observation by visual inspection carried out daily. The effects of DNA on the nucleation of protein crystals were studied by counting the number of drops with crystals.

The induction time was determined visually by optical microscopy, measuring the elapsed time between the droplet deposition and the appearance of the first visible crystal under an optical microscope (Olympus SZM-T4, Beijing, China) at a maximum magnification of 90 ×. Each set contained 48 samples and was repeated in triplicate to test the reproducibility of the result.

3. Results and Discussion

In this study, we conducted a series of experiments to explore if the addition of DNA as a polymeric additive has a positive effect on protein crystallization. We purchased three kinds of DNA from Sigma-Aldrich, the number of base pairs for all DNA were determined by 1% agarose gel electrophoresis (Figure S1), the results obtained as ~15,000 bp, ~1000 bp, and ~100 bp, respectively. Then, we estimated the molecular weights of DNA by taking an average number of 330 per base [35]. Therefore, three kinds of DNA with different molecular weights were used for comparison which were calf DNA (~9900 kDa), salmon DNA (~660 kDa), and herring DNA (~66 kDa). Lysozyme was chosen as a model protein due to its accessibility and abundance and has been widely used in previous studies [36–38]. In a typical experiment, lysozyme was dissolved in protein buffer (0.05 M NaAc, pH 4.6) to obtain lysozyme solution while DNA solutions can be prepared by dissolving three kinds of DNA into lysozyme precipitant buffer (1.0 M NaCl, 0.05 M NaAc, pH 4.6). The control experiment was conducted in parallel without the addition of DNA for comparison. An equal volume of lysozyme and DNA solution were used for crystallization. The daily crystal observation was conducted over a period of time until crystal growth reached a plateau. Conventional hanging-drop vapor diffusion technique was employed [39,40]. From daily observation, the crystal growth of lysozyme usually reached a plateau on the seventh day at 4 °C.

In this study, the degree of crystallizations was evaluated by crystal quantity denoted as crystal percentage, the percentage of crystallized drop. Higher difference of crystal percentage compared with control indicated a greater effect to enhance protein crystallization. Figure 1 demonstrated the percentage of crystallized drops with various concentrations of lysozyme (2.5–10 mg/mL) and DNA (0.1–20 mg/mL) on the seventh day (original data in Tables S1–S12). The control experiment without DNA addition was shown as the leftmost column for each lysozyme concentration. As depicted in Figure 1A, it was found that along *x*-axis, the percentage of crystallized drops increased with DNA concentration when calf DNA with molecular weight ~9900 kDa was utilized. At the lysozyme concentration of 2.5 mg/mL, the crystal percentage in average increased from 0% for blank sample to 2.8% and 33% with the presence of calf DNA at the concentration of 0.1 and 5.0 mg/mL, respectively. A similar trend was also observed for other lysozyme concentrations, showing that the crystal percentage increased proportionally with DNA concentration.

It is worth noting that the crystal percentage has reached a relatively constant value beyond both lysozyme concentration of 5.0 mg/mL and calf DNA concentration of 1.0 mg/mL, indicating the additional effect brought by DNA became less significant. A similar phenomenon was also observed for crystallization drops with salmon and herring DNA, but the upper threshold concentrations of protein and DNA were different. The enhanced effect of both salmon and herring DNA became limited beyond both DNA concentration of 10 mg/mL and protein concentration of 7.5 mg/mL, and below both values, we called it the 'effective window'. The determination of effective concentrations guided us to adjust DNA and protein concentration for protein crystallization. Single crystal X-ray crystallography on lysozyme crystals with DNA was carried out, and it showed that DNA at low concentrations had little effect on the quality of lysozyme crystals (Figures S2–S4). Combining all the observations, it was shown that the addition of DNA as polymeric additive has a positive effect on lysozyme crystallization at a certain effective window.

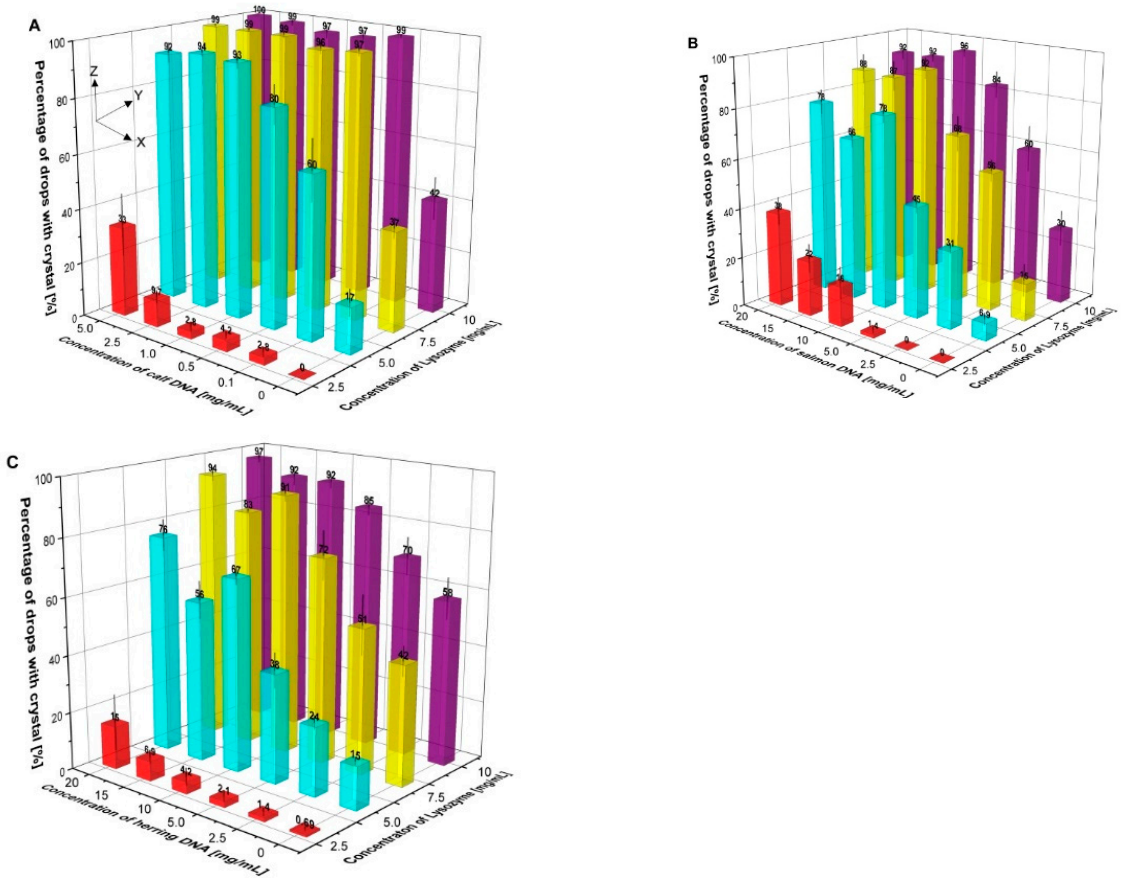

Figure 1. Crystallization success rate of lysozyme using (**A**) calf DNA (**B**) salmon DNA (**C**) herring DNA at 4 °C on the seventh day, respectively.

In addition, by further examination of Figure 2A–D, the effect of salmon and herring DNA on enhancement of lysozyme crystallization were similar, demonstrated by the similar percentage of crystallized drops, for instance, around 90% of drops crystallized for both salmon and herring DNA at lysozyme concentration of 10 mg/mL. This phenomenon may be contributed to their similar molecular weight for salmon and herring DNA which were ~660 kDa and ~66 kDa. Furthermore, the percentage of crystallized drop with calf DNA increased from herring DNA by 14.7-fold, 1.4-fold, 0.4-fold, and 0.2-fold at lysozyme concentration of 2.5, 5.0, 7.5, and 10 mg/mL, respectively. Therefore, the effect of different DNA on lysozyme crystallization was similar at higher lysozyme concentrations.

In terms of the induction time, Figure 2A showed that the crystal can be observed from the first day for calf DNA compared to zero crystal formed for blank sample, indicating the presence of calf DNA drastically reduced the induction time of crystal growth. As for salmon and herring DNA, their effect was less noticeable at a lysozyme concentration of 2.5 mg/mL as their induction time was similar as that of control sample. At higher lysozyme concentrations in Figure 2B–D, the additional effect of all DNA on induction time was little. Therefore, the effect of DNA was more pronounced at low lysozyme concentration of 2.5 mg/mL. Low protein concentration is more beneficial to biological and pharmaceutical industry due to lower protein consumption and lower economic costs. Overall, calf DNA with the highest molecular weight exerts the greatest influence on both success rate and induction time of lysozyme crystallization.

Combining all the above observations, it is concluded that the addition of DNA has a positive effect on the success rate and induction time of lysozyme crystallization as well as the effect is more pronounced for DNA with higher molecular weight. We proposed that the addition of DNA to lysozyme solution possibly caused an excluded-volume effect, which induced depletion attraction. The depletion force may be described as effective attraction between two protein molecules in a

crowded solution induced by the volume occupied by DNA between protein molecules [41,42]. Moreover, the DNA molecules possibly interacted with water molecules in the solution causing the change of surface tension of water and less water molecules surround protein molecules [43], resulting in higher local protein concentration and lower supersaturation concentration to enhance protein crystallization. The competition of water molecules between DNA and protein also likely kept fewer water molecules from accumulating around protein molecules [44], resulting in higher local protein concentration as well as lower supersaturation concentration to promote protein crystallization. DNA may also serve as a nucleant for heterogeneous crystal nucleation. In plain words, the biological compatibility and biochemical similarity facilitate adsorption of lysozyme on DNA, thus promoting crystal nucleation. The largest DNA, which is calf DNA, renders more nucleation-promoting centers on its surface. The higher chain length of DNA with higher molecular weight was likely to provide a greater competition effect of volume and water with protein molecules, resulting in a higher protein local concentration. Herein, calf DNA with the highest molecular weight among all three DNAs has the highest potential to promote protein crystallization.

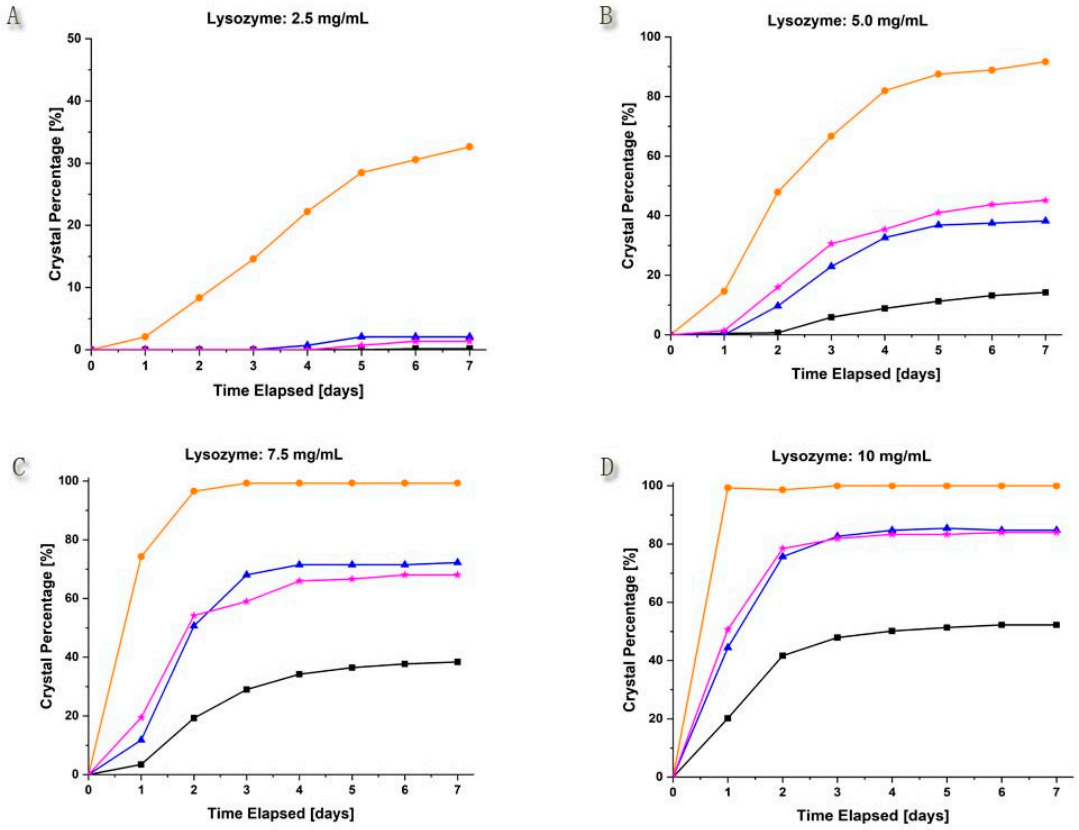

Figure 2. Crystal percentage over a period of 7 days with 5.0 mg/mL calf DNA (orange line with dots), 5.0 mg/mL salmon DNA (pink line with stars), 5.0 mg/mL herring DNA (blue line with triangles) and Blank (black line with square) at lysozyme concentration of (**A**) 2.5 mg/mL, (**B**) 5.0 mg/mL, (**C**) 7.5 mg/mL, and (**D**) 10 mg/mL at 4 °C.

We made a final observation of lysozyme crystal numbers and sizes with different kinds and concentrations of DNA. As shown in Figure 3, the use of calf DNA as a polymeric additive generated the formation of the highest number of crystals per drop, because of an increase in nucleation rate. At the same time, both salmon and herring DNA resulted in crystals with larger sizes but the crystal number per drop remained relatively constant over crystal growth. Moreover, the effect was more pronounced especially at higher DNA concentrations (Figure 3D–F). This further verifies that DNA can serve as an effective polymeric material to enhance protein crystallization.

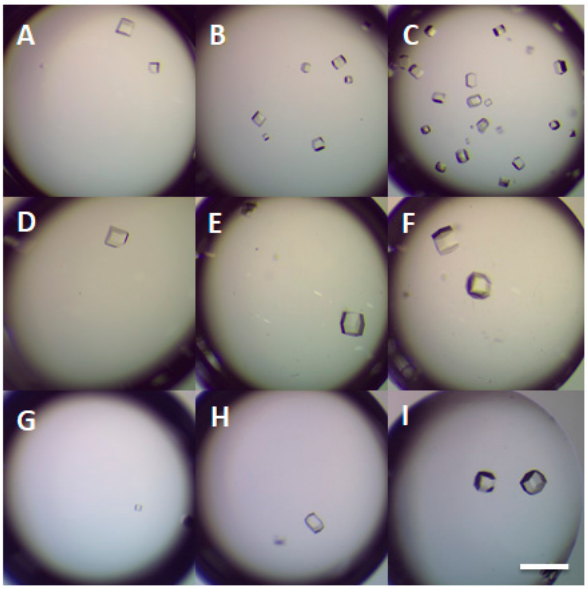

Figure 3. A typical set of crystal pictures with 10 mg/mL lysozyme on the first day using (**A**) 0 mg/mL calf DNA; (**B**) 1.0 mg/mL calf DNA; (**C**) 5.0 mg/mL calf DNA; (**D**) 0 mg/mL salmon DNA; (**E**) 10 mg/mL salmon DNA; (**F**) 20 mg/ mL salmon DNA; (**G**) 0 mg/mL herring DNA; (**H**) 10 mg/mL herring DNA; and (**I**) 20 mg/mL herring DNA (scale bar: 600 μm).

4. Conclusions

On the whole, we presented for the first time using DNA as a new kind of polymeric additive to enhance protein crystallization. The success rate of lysozyme crystallization was enhanced with the presence of all three DNAs, especially at low protein concentration. Among the three kinds of DNA, the presence of calf DNA with the highest molecular weight showed the greatest improvement in lysozyme crystallization. In this work, the influence of DNA on protein crystallization at low protein concentrations has highlighted the outstanding potential of DNA as a novel additive for systems with limited amounts of protein. Considering the programmable and designable characteristics of DNA, specific DNA with well-defined sequence and length can be synthesized, thus, it is envisioned that DNA has excellent potential to improve systems where biological molecules are difficult to crystallize, as well as to maximize the efficiency of using scarce resources. This finding is only the starting point, the use of lysozyme can be extended to other proteins. In view of the short history of using DNA as polymer starting 30 years ago, As the field is becoming more mature, it will open up a new platform and promote promising applications in material science, detection, drug delivery, and tissue engineering.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4352/9/4/186/s1, Figure S1: 1% agarose gel determination of base pair numbers for (a) herring DNA (b) calf DNA and salmon DNA, respectively; Figure S2: X-ray diffraction of lysozyme crystals with herring DNA; Figure S3: X-ray diffraction of lysozyme crystals with salmon DNA; Figure S4: X-ray diffraction of lysozyme crystals with calf DNA; Table S1: Number of drops with crystals by adding calf DNA at 2.5 mg/mL lysozyme; Table S2: Number of drops with crystals by adding calf DNA at 5.0 mg/mL lysozyme; Table S3: Number of drops with crystals by adding calf DNA at 7.5 mg/mL lysozyme; Table S4: Number of drops with crystals by adding calf DNA at 10 mg/mL lysozyme; Table S5: Number of drops with crystals by adding salmon DNA at 2.5 mg/mL lysozyme; Table S6: Number of drops with crystals by adding salmon DNA at 5.0 mg/mL lysozyme; Table S7: Number of drops with crystals by adding salmon DNA at 7.5 mg/mL lysozyme; Table S8: Number of drops with crystals by adding salmon DNA at 10 mg/mL lysozyme; Table S9: Number of drops with crystals by adding herring DNA at 2.5 mg/mL lysozyme; Table S10: Number of drops with crystals by adding herring DNA at 5.0 mg/mL lysozyme; Table S11: Number of drops with crystals by adding herring DNA at 7.5 mg/mL lysozyme; Table S12: Number of drops with crystals by adding herring DNA at 10 mg/mL lysozyme.

Author Contributions: B.Z. was involved in all stages of the work, including conducting experiments and analyzing the data; S.T. and V.T. did parts of experiments; P.L. and S.F. helped analyze the data; B.Z., Y.W., and Z.Y. wrote and revised the paper; L.X., Z.Y., and J.Y.Y.H. acted as the supervisors and work planning.

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Conflicts of Interest: The authors declare no conflict of interest.

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