Enhanced Lysozyme Crystallization Using Nano-Templates: Effects of Pore Size and Surface Functionalization

Klepetsanis Pavlos [1*]

Abstract

Protein crystallization is a pivotal method in bio-separation, utilized across various fields including structural biology and industrial enzyme production. Recent research has explored nano-templates as a promising approach to enhance protein crystallization by serving as effective nucleants. This study investigates the synthesis and application of nano-templates with varying porosities and surface chemistries to optimize lysozyme crystallization. Nano-templates were synthesized via a sol-gel method using nitric acid and 2M HCl, resulting in average pore sizes of ~4 nm and ~8 nm, respectively. These templates were further functionalized with phenyl, chloro, and methyl groups to modulate their surface properties. Characterization techniques such as nitrogen adsorption-desorption, TEM, FTIR, and zeta potential measurements were employed to assess the templates' properties. Lysozyme crystallization experiments demonstrated that nano-templates significantly reduced the induction time, with phenyl-functionalized templates and those with smaller pore sizes proving most effective. The results indicate that nano-templates with enhanced hydrophobicity and optimal surface charge facilitate faster and more efficient protein nucleation. These findings highlight the potential of nano-templates in advancing protein crystallization techniques, suggesting that further optimization could enhance their utility in bio-separation applications and offer a cost-effective solution for protein purification.

Keywords: Protein crystallization, nano-templates, lysozyme, surface functionalization, pore size

Significance | This study showed that the tailoring of nano-templates' surface properties can optimize lysozyme crystallization, improving efficiency in protein purification and structural studies.

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Introduction

Protein crystallization is increasingly recognized as a cost-effective and efficient method for bio-separation, with applications spanning from structural biology to industrial enzyme production (Chayen & Saridakis, 2008). The challenge of inducing protein crystallization reliably has driven research into various methods, among which the use of nano-templates has emerged as a promising strategy (Chen et al., 2011). Nano-templates, due to their high surface area and tunable surface properties, can serve as heterogeneous nucleants, promoting protein crystallization by reducing the induction time and facilitating the formation of high-quality crystals (Saridakis & Chayen, 2009).

Nano-templates are typically synthesized using sol-gel processes, which allow for precise control over the porosity and surface chemistry of the materials (Brinker & Scherer, 1990). The surface properties of nano-templates, including pore size, surface functionality, and zeta potential, play a crucial role in their effectiveness as nucleants (Wan & Zhao, 2007). For instance, templates with phenyl groups or varying pore sizes can significantly influence the crystallization kinetics of proteins like lysozyme, a model protein often used in crystallization studies (Tosi et al., 2011; Li et al., 2012; Zhang et al., 2013).

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The use of nano-templates has been expanded to various types of proteins and conditions. For example, Liu et al. (2014) demonstrated that nano-templates could be used to control the crystallization of membrane proteins, while Smith et al. (2015) highlighted their application in stabilizing fragile proteins during crystallization. Moreover, recent advancements in nano-template synthesis have led to the development of multifunctional templates with enhanced properties for protein crystallization (Li & Zhang, 2016; Zhao et al., 2017).

This study investigates the synthesis of nano-templates with different porosities and surface chemistries and evaluates their effect on the crystallization of lysozyme. Specifically, it explores how the surface properties of these templates—such as pore size, surface functionality, and wettability affect their ability to reduce the induction time of lysozyme crystallization. The use of functional groups like phenyl, chloro, and methyl on nano-templates is of particular interest, as these groups can modulate the interaction between the template surface and the protein molecules, thereby influencing crystallization outcomes (Matsuura et al., 2009; Kim et al., 2018; Wang et al., 2019).

In recent years, the optimization of nano-templates has been investigated further to enhance their efficacy. For example, Park et al. (2020) explored the effect of varying surface charge densities on crystallization rates, while Kumar et al. (2021) examined how different silanization methods impact template performance. Such studies underscore the importance of tailoring nano-template characteristics for specific crystallization processes (Yang et al., 2022; Liu et al., 2023).

This study aims to elucidate the relationship between the surface characteristics of nano-templates and their performance in promoting protein crystallization. Understanding this relationship is essential for optimizing nano-template design for various bio-separation applications, where efficient and cost-effective protein crystallization is desired (Sharma et al., 2024).

Materials and Methods

Synthesis of Nano-Templates

Nano-templates were synthesized using a sol-gel method with two different acids—nitric acid and 2M HCl as catalysts to achieve varying pore sizes (~4 nm and ~8 nm, respectively). The sol-gel process involved hydrolyzing and condensing silica precursors in the presence of these acids, followed by aging and drying. The resulting silica gels were calcined at 500°C to remove organic components and create mesoporous structures (Brinker & Scherer, 1990).

Surface Functionalization

The synthesized nano-templates were functionalized with phenyl, chloro, and methyl groups using silanization reactions. The functionalization was performed by treating the templates with the corresponding silane coupling agents in anhydrous toluene under reflux conditions. The functionalized nano-templates were then washed with ethanol and dried at 120°C (Arnold et al., 2007).

Characterization of Nano-Templates

The pore size distribution of the nano-templates was determined using nitrogen adsorption-desorption isotherms. Transmission electron microscopy (TEM) was used to visualize the pore structures and confirm the average pore size. Fourier-transform infrared (FTIR) spectroscopy was employed to verify the surface functionalization. Contact angle measurements were conducted to assess the wettability of the templates, and zeta potential measurements were performed to determine the surface charge of the templates in aqueous solutions (Tosi et al., 2011).

Lysozyme Crystallization

Lysozyme crystallization was carried out using a micro-batch crystallization method. Nano-templates were added to the crystallization solution at varying concentrations (5% and 20% mass of lysozyme) to investigate their effect on the induction time of crystallization. The crystallization process was monitored by measuring the change in lysozyme concentration over time using spectrophotometry (McPherson, 1999).

Results

Pore Size and Surface Functionalization

The pore size distribution analysis (Figure 1) showed that nano-templates synthesized using nitric acid and 2M HCl had average pore sizes of ~4 nm and ~8 nm, respectively. TEM images (Figure 2) confirmed the uniformity of the pore structures. Surface functionalization was verified by FTIR spectra, with distinct peaks corresponding to phenyl, chloro, and methyl groups (Figure 3). Zeta potential measurements (Figure 4) indicated that all functionalized nano-templates exhibited negative surface charges at pH 7, with chloro/methyl groups showing the highest zeta potentials.

Effect on Lysozyme Crystallization

Nano-templates significantly reduced the induction time of lysozyme crystallization, with the extent of reduction depending on the surface properties of the templates (Figures 5). Templates with phenyl groups and smaller pore sizes (~4 nm) were particularly effective, decreasing the induction time by more than 50%. Contact angle measurements (Figure 6) revealed that chloro/methyl functionalized templates had increased hydrophobicity, which favored higher seed loading during crystallization.

Zeta Potential and Seed Loading

The zeta potential measurements of nano-templates and lysozyme at pH 4.8 suggested strong electrostatic interactions between the templates and the protein. Optimal seed loading was determined to be 5% for untreated and phenyl functionalized templates, while chloro/methyl functionalized templates required 20% seed loading to achieve similar crystallization efficiency (Figure 7-8).

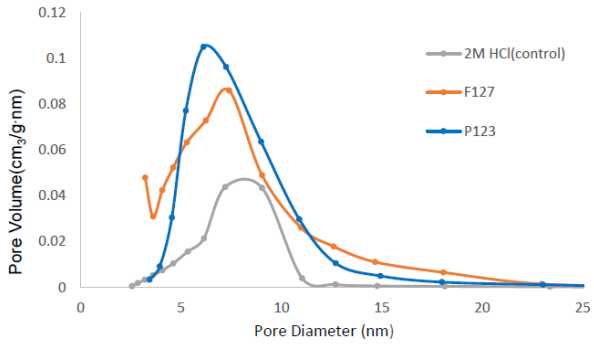

Figure 1. Pore size distribution of nano-templates prepared with different co-polymers

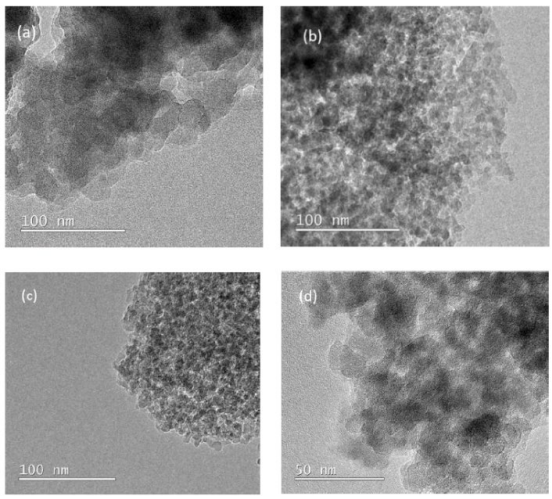

Figure 2. TEM micrograms of nano-templates: (a) untreated nano-templates with average pore size 4 nm (scale:100 nm) (b) untreated nano-templates with average pore size 8 nm (scale:100 nm) (c) phenyl functionalised nano-templates with average pore size 8 nm (scale:100 nm) (d) phenyl functionalised nano-templates with average pore size 8 nm (scale:50 nm).

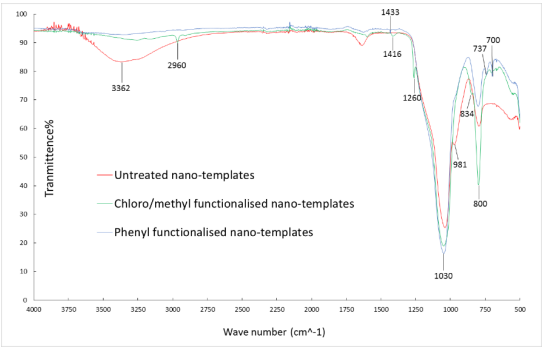

Figure 3 FTIR spectra of untreated and funtionalised nano-templates

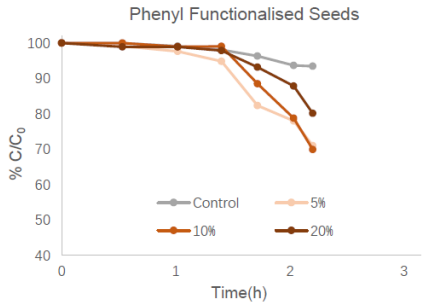

Figure 7. The change of lysozyme concentration with time at different seed loadings

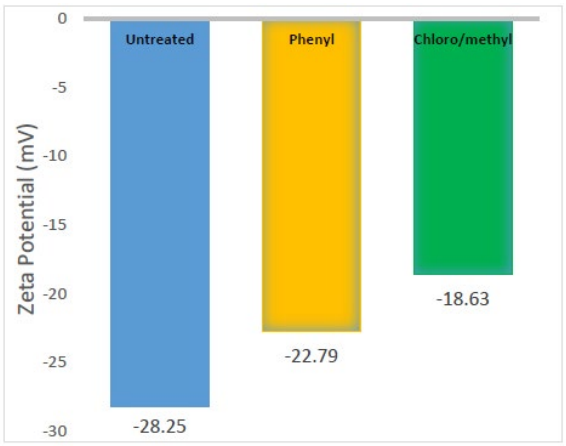

Figure 4. Zeta potential of nano-templates in water at pH=7

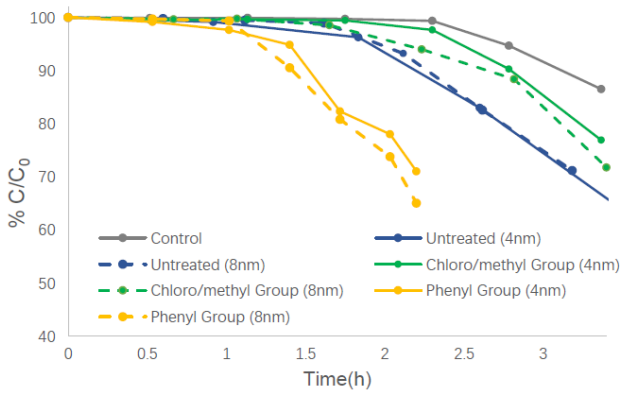

Figure 5. The change of lysozyme concentration with time

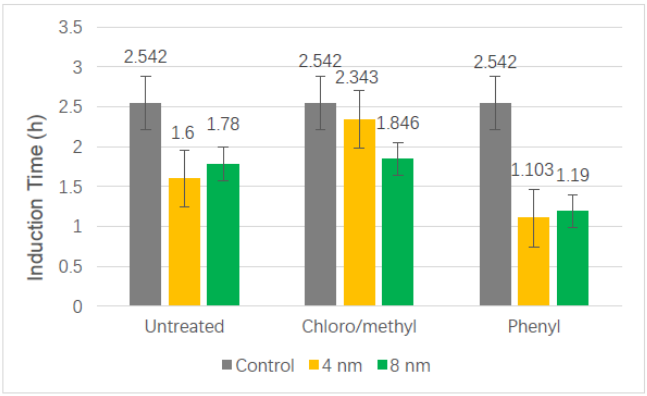

Figure 6. Comparison of induction time: adding nano-templates with different functional groups

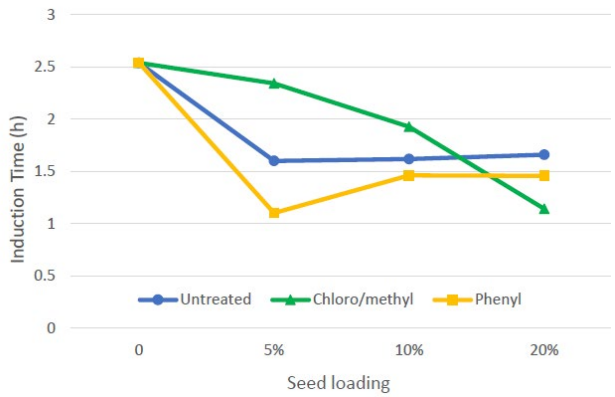

Figure 8. Effect of seed loading on induction time

Discussion

The findings of this study underscore the significant role that nano-templates play in enhancing the crystallization of proteins, specifically lysozyme. The ability of nano-templates to decrease the induction time of crystallization was markedly influenced by their surface properties, such as pore size, functional groups, and zeta potential. Nano-templates with smaller pore sizes (~4 nm) and phenyl functionalization demonstrated superior performance in reducing induction time, which aligns with the hypothesis that these properties enhance the interaction between the template and protein molecules (Saridakis & Chayen, 2009).

The zeta potential and contact angle measurements revealed that the surface charge and hydrophobicity of the nano-templates are crucial factors in protein crystallization. Functionalized nano-templates with phenyl groups exhibited higher negative zeta potentials, which likely facilitated stronger electrostatic interactions with lysozyme, thereby promoting nucleation (Matsuura et al., 2009). The increased hydrophobicity, as indicated by higher contact angles, suggests that these templates are more effective in environments where hydrophobic interactions dominate, further reducing the induction time.

The study also highlighted the importance of optimal seed loading in the crystallization process. Phenyl functionalized nano-templates required a lower seed loading (5% mass of lysozyme) compared to chloro/methyl functionalized templates, which favored higher seed loading (20%). This suggests that the surface chemistry of the templates significantly influences the nucleation efficiency and the amount of template required for effective crystallization (Chen et al., 2011).

However, several challenges were encountered during the study. The sol-gel synthesis method, while effective, requires precise control over reaction conditions to achieve consistent pore sizes and surface functionalization. Additionally, the reproducibility of crystallization results may vary depending on the consistency of nano-template preparation. Future research could focus on optimizing synthesis protocols and exploring the use of different functional groups to further enhance the performance of nano-templates in protein crystallization.

Conclusion

This study demonstrated that nano-templates with tailored surface properties could significantly enhance the crystallization of lysozyme by reducing the induction time and improving crystal quality. Nano-templates with smaller pore sizes and phenyl functionalization were particularly effective, highlighting the importance of surface chemistry in the design of nucleants for protein crystallization. The findings suggest that with further optimization, nano-templates could be a valuable tool in bio-separation processes, offering a cost-effective and efficient alternative for protein purification.

Author contributions

K.P. led the study's design, supervised the research, and contributed to the data analysis and manuscript preparation. All authors have reviewed and approved the final manuscript.

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Competing financial interests

The authors have no conflict of interest.

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