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External Fields as Control Strategies to Promote Biomolecule Nucleation



Rohan P. Y. van Tooren and Hüseyin Burak Eral

Contents

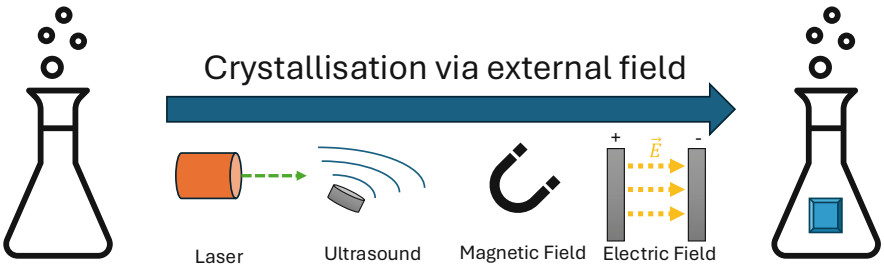
1 Introduction 252
2 Non-photochemical Laser-Induced Nucleation 253
2.1 Historical Perspective 253
2.2 Experimental Observations 255
2.3 Theories 256
2.4 Protein NPLIN 260
2.5 Design Considerations 262
3 Other Field Control Techniques 264
3.1 Other Light Irradiation-Based Techniques 264
3.2 Electric Fields 264
3.3 Magnetic Fields 265
3.4 Ultrasound 265
4 Conclusion and Outlook 265
References 267

Abstract Acoustic, electric, and magnetic external fields have been extensively investigated as a promising non-invasive approach to control nucleation from solution. Among these approaches, light fields leveraging lasers such as non-photochemical laser-induced nucleation (NPLIN) have seen a recent surge of interest. NPLIN involves an interaction between the solute-solvent system and laser light, but takes place at wavelengths and intensities not known to invoke any photochemical phenomena. Promising applications include direct enhancement of the nucleation rate, as well as providing polymorphic control and spatial precision in initiating crystallisation. This chapter mainly reviews the current state of NPLIN research focusing on biomolecules. Secondly, this chapter discusses other external-field-based methods, albeit in much less detail. The chapter concludes with the

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authors' perspective on future directions of research dedicated towards biomolecule crystal nucleation using such field-based methods.

Graphical Abstract



Keywords Electric field-assisted crystallisation, Magnetic field-assisted crystallisation, Non-photochemical laser-induced nucleation, Sonocrystallisation

Nomenclature

Symbols for Mathematical Equations

I	Laser intensity
P_{nuc}	Nucleation probability
m	Proportionality constant

Abbreviations

DP	Dielectric polarisation
HEWL	Hen egg-white lysozyme
IH	Impury heating
NPLIN	Non-photochemical laser-induced nucleation
OKE	Optical kerr effect
Phe	Phenylalanine
Trp	Tryptophan
Tyr	Tyrosine

1 Introduction

As discussed in previous chapters, nucleation of biomolecules is an out-of-equilibrium process that is challenging to control. Consequently, various strategies for controlling biomolecule nucleation have been investigated. This chapter focuses

on techniques that employ external fields—specifically acoustic and electromagnetic fields. These methods offer several potential advantages:

1. Spatiotemporal control: Most methods discussed in this chapter are implemented via devices that can project external fields in a consistent and directional manner and can be activated or deactivated on demand. These features make it possible, in principle, to initiate nucleation with high spatial resolution and precise timing.
2. Variable intensity: The external field generators can typically be electronically controlled to deliver a range of power outputs, enabling modulation of the nucleation-inducing effect.
3. Non-invasiveness: These methods do not involve the introduction of additional chemical or biological species, making them particularly suitable for crystallisation processes requiring highly clean environmental conditions.

The use of external fields to control nucleation is a relatively recent development in the field of biomolecular crystallisation. Although each of the techniques discussed has its own independent research history, most of them emerged in the late twentieth century [1–4]. The bulk of scientific research, including review articles on individual techniques [5–8], began to appear in the early twenty-first century.

In this chapter, we will examine methods involving electric fields, magnetic fields, light, and ultrasound. Given the authors' primary expertise in light-based methods and specifically non-photochemical laser-induced nucleation (NPLIN), these will be discussed in more detail. The chapter then proceeds with a more concise treatment of the remaining approaches, followed by a discussion of overarching principles and future directions for research in this area.

2 Non-photochemical Laser-Induced Nucleation

2.1 Historical Perspective

Non-photochemical laser-induced nucleation (NPLIN) was discovered serendipitously in 1996 by Garetz et al. [1]. In their work published in 1996, millilitre-sized vials with aqueous supersaturated urea solutions in them were exposed to laser light. When left undisturbed, these supersaturated urea solutions nucleate over weeks, and instead, after exposure to the laser, they nucleated instantly, see Fig. 1. Since the solute did not have any absorption peaks at the corresponding wavelength (1.06 μm), and the laser intensity supplied was significantly below the optical breakdown threshold, the authors concluded that photochemical effects were unlikely. The name "Non-Photochemical Laser-induced Nucleation" (NPLIN) was derived from this conclusion. In later work on aqueous supersaturated glycine solutions [10, 11], it was observed that linear polarised light favoured γ-phase glycine formation, whereas circular polarised light favoured the formation of α-phase glycine. These two promises of spatiotemporal control and polymorph selectivity presumably sparked widespread interest in the field over time.

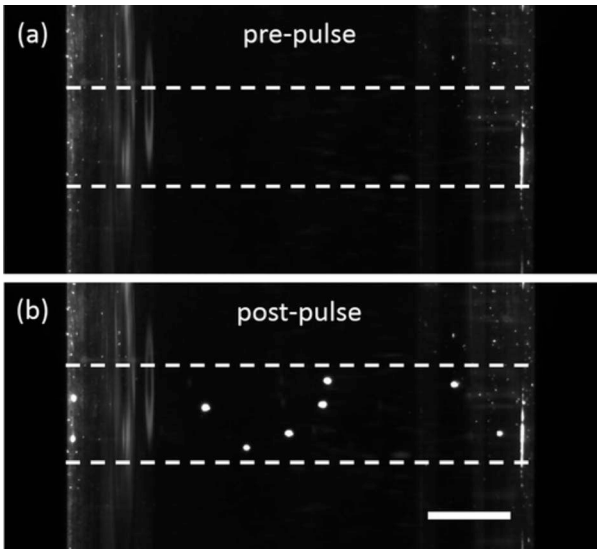


Fig. 1 NPLIN in action. (**a,b**) Unfiltered NH4Cl solution before (**a**) and 1.6 seconds after (**b**) laser irradiation. The laser path is indicated by the dashed lines [9]

NPLIN research has since been conducted successfully on protein crystallisation. Hen egg-white lysozyme (HEWL) was successfully crystallised using NPLIN by Adachi et al. [12] in 2003. Yennawar et al. [13] conducted a study involving NPLIN of several proteins (ribonuclease B, glucose dehydrogenase, lysozyme, sorbitol dehydrogenase, fructose dehydrogenase, and myoglobin) in 2010. Iefuji et al. [14] crystallised glucose isomerase in 2011. To the authors' knowledge, however research using biomolecules beyond glycine and urea has since been stagnant. Most research from there was conducted on smaller organic and inorganic compounds, including metal halides [15], CO_2 gas [16, 17], and single-component systems [18, 19]. The authors conjecture the reason for this is twofold:

1. The mechanism of NPLIN was a mystery at the time, and it was uncertain to what extend ageing, degradation, and laser polarisation had to be considered. Thus, until further fundamental insights, applications were possible, but engineering was not. Given the already arduous process of obtaining statistically significant evidence on NPLIN experiments and the long waiting times involved in protein crystallisation, the former might not have been attractive without a promise of engineering precision at later design stages.
2. Progress in understanding NPLIN was achieved by testing compounds for which competing hypotheses would yield different outcomes. These test compounds were not proteins, but metal halides and dissolved gases. Therefore, fundamental NPLIN research in later years did not focus on proteins.

However, a lot has happened in the NPLIN research field since then. The next two subsections discuss some of this research, which is intended to quickly cover the state of the art of NPLIN. The authors provide only a brief account of this on NPLIN

in general, to focus more on the prospects for biomolecules. There are recent review papers available on NPLIN as a field in general, see Alexander et al. [20] or Korede et al. [6].

2.2 Experimental Observations

Experimentally, NPLIN research tends to focus first on whether NPLIN is possible for a given system and then possibly quantify this by recording the "NPLIN probability," which signifies what percentage of irradiated samples crystallised after a given resting time.[1] This resting time varies per experiment (e.g. chemicals involved, supersaturation), but is always set such that spontaneous nucleation is very small and ideally negligible. There have been numerous observations on the experimental side, and a few of them will be highlighted here:

1. NPLIN observed in a broad set of solute/solvent systems: NPLIN has been observed with many chemically distinct solutes, including metal halides [15] and small organic molecules [11, 21, 22], proteins [13, 23], and dissolved gases [16, 17]. However, not all solutions undergo NPLIN: Ward et al. [9] observed that acetamide does not undergo NPLIN, despite the structural similarity to urea, of which NPLIN has been observed [11]. In Barber's doctoral thesis [24], it was reported that sodium chlorate was also not affected by NPLIN.[2]
2. Polymorph control via laser polarisation: For several organic compounds such as glycine [11], L-histidine [21], carbazepima [25], and sulfathiozole [26], it was reported that laser polarisation affected the final polymorph distribution of nucleated samples. However, this observation was not reproduced for glycine in later work by Liu et al. [27].
3. Positive correlation in supersaturation and laser intensity [28, 29].
4. Existence of a threshold laser intensity: It is reported by various authors that there is a laser intensity threshold below which NPLIN is not observed [1, 22, 30]. This laser intensity threshold depends on the solute, wavelength, and ambient temperature [15, 22]. For instance, the laser intensity threshold of urea is an order of magnitude higher than that for KCl: $\sim 50\ \mathrm{MW/cm^2}$ [22] vs. $\sim 3\ \mathrm{MW/cm^2}$ [15].
5. Effect of impurities and nanoparticles: Ward et al. [9] studied the effect of filtration and nanoparticle doping on NPLIN probability. They reported that filtration of supersaturated solutions decreased the NPLIN probability. The

[1] It is relevant to note that this standardisation of quantifying NPLIN via statistics is less mature than in crystallisation at large, where induction times can be quantified. There have been efforts to define for instance the "lability" [20] which captures the sensitivity of NPLIN to laser intensity. However, this procedure is far from standardised, and the connection to first principles is as yet disputable.

[2] It may be worth mentioning however that this observation was in the context of the standard NPLIN experiment involving a millilitre-sized vial and an unfocused laser. Perhaps a focused laser with higher laser intensity would yield a different outcome.

addition of Fe_2O_3 nanoparticles of hydrodynamic diameter 750 nm to filtered solutions increased the observed NPLIN probability. With a microfluidic setup enabling NPLIN experiments with 100 μm droplets allowing for stronger statistics (thousands of repetitions), Korede et al. [31] observed decreased NPLIN probability with filtration and increased NPLIN probability with the addition of Fe_2O_3 nanoparticles. In Korede's doctoral thesis [32], it was found that the NPLIN probability of filtered samples was the same as that of control experiments without laser irradiation, and that incremental increases in gold nanoparticle dopant concentration lead to higher NPLIN probabilities. They also found that silica nanoparticles lead to lower NPLIN probabilities compared to gold-based ones. Nagalingam et al. [29] added a soluble $O(10)ppm$ level impurity, $KMnO_4$. With this system, it was observed that NPLIN probability scaled with increasing supersaturation and laser intensity. Furthermore, they observed a thermocavitation bubble microseconds after laser irradiation using high-speed imaging. The maximum radius of this bubble correlated with the NPLIN probability observed.

These observations have been crucial in the formulation of theories regarding NPLIN, as will be discussed in the upcoming theories section.

2.3 *Theories*

The authors discuss only the mainstream theories formulated for NPLIN. They are summarised in Fig. 2. The mathematics proposed with regards to each mechanism are left outside the scope of this brief summary. The reader is invited to read respective detailed mathematical discussions in other papers. These are Sun et al. [11] for the optical Kerr effect, Alexander et al. [20] for the dielectric polarisation, and Hidman et al. [33] for impurity heating (Table 1).

The first mechanism proposed is the optical Kerr effect (OKE) [34, 35]. The OKE specifically refers to the tendency of particles to align themselves with the polarisation of a laser field, see Fig. 2a. It is hypothesised that then this local alignment reduces the Gibbs free-energy barrier of nucleation, leading to a promoted nucleation rate [1].

Several problems have been identified with regards to this theory however:

1. The estimated alignment changes due to laser axis polarisation are too small to believably affect the nucleation rate. It was later suggested that cooperative effects between solute molecules could lead to higher values than estimated. Potts lattice gas Monte Carlo simulations by Knott et al. [36] however estimated this cooperative effect to be too weak to explain the alignment of solute molecules in the time frame of a laser pulse.
2. The initial reason for involving OKE was the claimed observation of polarisation alignment sensitivity [1]. It should however be mentioned that the means by

Table 1 Observation and mechanism summary for NPLIN (inspired by Barber [24] and Korede [6]). OKE = optical Kerr effect, DP = dielectric polarisation, IH = impurity heating. Meaning of symbols: ✓—can be explained; x—cannot be explained; ?—needs more insight/analysis

	Observation		OKE	DP	IH
1	NPLIN is reported for a broad range of systems	Small organics	✓	✓	✓
		Metal halides	x	✓	✓
		Gases	x	x	✓
2	Polarisation Dependence		✓	✓	?
3	Polymorph control		✓	x	?
4	Dependence laser intensity and supersaturation		✓	✓	✓
5	Threshold laser intensity		x	x	✓
6	Effect of impurities and nanoparticles		x	x	✓

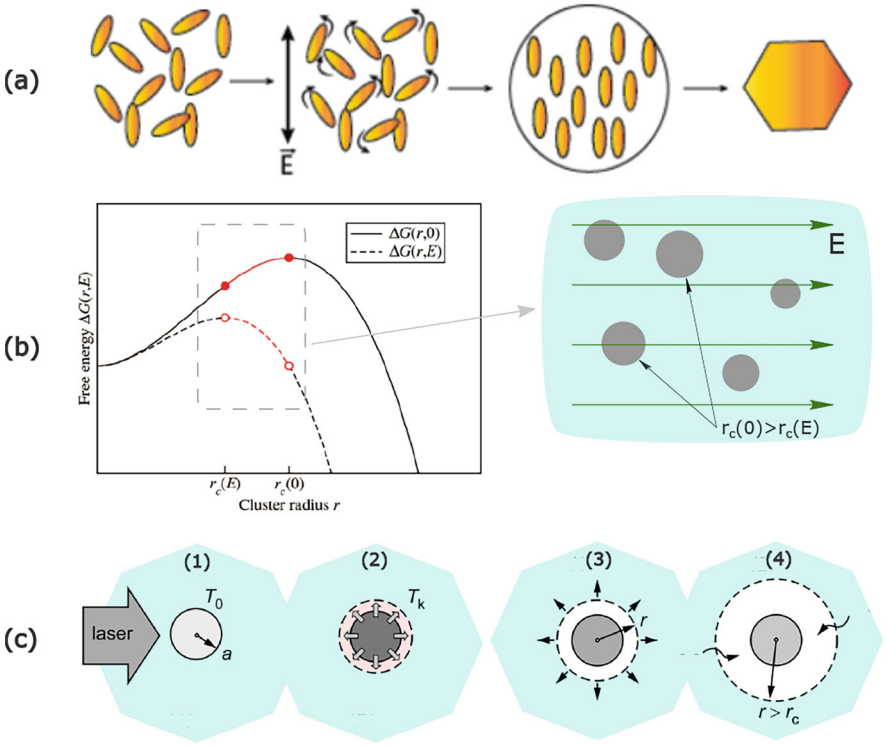


Fig. 2 Plausible mechanisms for NPLIN. (**a**) Optical Kerr effect: an external electric field torques particles into a more aligned state, favouring nucleation. (**b**) Dielectric polarisation: solute preclusters are stabilised by an applied electric field, lowering the barrier to nucleation [17]. (**c**) Impurity heating: a tiny impurity particle is heated by laser light, leading to local heating and evaporation of light, and thereby concentration and supersaturation of solute particles, leading to nucleation. (Adapted from Korede et al. [6], reprinted under the conditions of license type CC-BY 4.0)

which this polarisation alignment was observed are not mentioned in the article. Furthermore, the observation was not reproduced in later work by Liu et al. [30].
3. OKE also fails to explain the observation of a laser intensity threshold below which no NPLIN occurs.
4. OKE cannot explain why some systems without a polarisation axis, such as KCl, undergo NPLIN.

The doubts surrounding the OKE-based model in turn led to the proposal of the dielectric polarisation (DP) model. This model attempts to explain the NPLIN of metal halides in terms of the difference in dielectric permittivity between solute clusters and the bulk solution. Here, it is hypothesised that isotropic polarisation of preclusters of nuclei—as per those imagined in a two-step nucleation process—leads to a reduction in the precluster's free energy, thereby stabilising it, see Fig. 2b. The important difference with the OKE here is that OKE depends on differences in polarisability within the solute, whereas with DP the dependence is on the difference in polarisability between the solute and the solvent. The model ultimately predicts a Poisson-type dependence of nucleation probability in laser intensity:

$$P_{nuc} = 1 - e^{-mI}, \tag{1}$$

where m is a proportionality constant.

The above expressions correctly predict a positive progressing correlation between laser intensity and crystals observed, and more specifically the linear trend observed at low laser intensities [15]. There are two significant problems with the DP model:

1. The model depends on the permittivity of the solute precluster being higher than that of the solution. Therefore, the model cannot explain the NPLIN of dissolved gases which has been observed in work by Ward et al. [17] on CO_2 dissolved in water and aqueous sucrose solutions.
2. The DP model cannot explain the observation of an intensity threshold I_0 below which no NPLIN takes place: Even an infinitesimally small laser field would reduce the nucleation barrier in this model. To address this shortcoming in particular, an impurity heating-based mechanism was proposed.

The impurity heating (IH) mechanism aims to explain the appearance of a threshold intensity I_0 by employing a dependence on gas formation. The hypothesis is that the laser heats up small particles suspended in the liquid. These heated particles in turn exchange heat with the surrounding liquid, which evaporates leading to bubble formation. This local evaporation thereby then leads to a local accretion of solute particles, increasing the supersaturation, and hence leading to higher observed nucleation probabilities. See Fig. 2c for a graphical illustration. Crucially, bubble formation requires a threshold value of supplied thermal energy, which can provide the missing link with the observed threshold laser intensity I_0 in NPLIN experiments.

The IH mechanism is supported by the observed dependence on impurities and nanoparticles named earlier. Furthermore, Nagalingam et al. [29] found evidence of NPLIN on KCl solutions only when a μs timescale bubble event could be observed. Using finite element simulations, they estimated an increased supersaturation near the transient vapour-liquid interface generated by the laser. The increased supersaturation was deemed sufficient for primary nucleation during the bubble's lifetime. However, there are some important details in these experiments with regards to method: In Nagalingam et al. [29]'s experiment, a focused laser was used and a soluble impurity $KMnO_4$ was added. Both of these may lead to conditions significantly different from those of classic NPLIN vial-based experiments as in Garetz et al. [1]'s first publication:

1. A dissolved impurity heated by a focused laser can be interpreted as heating a volume element of size determined by diffraction limitations, whereas a solid particle heated by an unfocused laser is more accurately interpreted as surface heating, where the surface area is not determined by optics but by the particle's geometric size. Surface heating might lead to directional bubble growth, whereas volumetric heating leads to spherical growth, and it is not yet investigated whether this could affect NPLIN.
2. Unfocused lasers experiments involving suspended solid particles can involve many individual bubble formation events which may or may not interact, whereas a single focused laser heating a dissolved impurity is in principle an isolated event. It is not known whether individual bubble formation events could change the presumed nucleation process.
3. In practice, focused lasers lead to way higher laser intensities. Higher laser intensities are correlated with higher NPLIN probabilities [28] but may perhaps include also a mechanistic change. For instance, high enough laser intensities may yield plasma formation [6] rather than vapour formation. It is not known whether nucleation in the vicinity of plasma is different with respect to vicinity of gas.

All of these concerns may have to be addressed in future work. But perhaps the most important concern regarding the matter of unfocused versus focused lasers is whether unfocused lasers can actually produce bubbles. Bubble formation is the most essential feature of the IH mechanism.

Recently, Barber et al. [37] investigated this question. In their experiments, they immerse a supersaturated cesium chloride solution in an oil bath. They use a continuous-wave laser to illuminate the setup, and a pulsed unfocused laser to induce NPLIN events. They then track the changes in the system with high-speed cameras. They observe crystal formation even with an unfocused laser. They also observe bubbles in both the inner aqueous solution and the outer oil bath. While the bubbles neighbouring crystal formation in the aqueous solution were too small to follow at high speed, the larger bubbles originating from dust at the inner oil-glass interface showed oscillations reminiscent of the Rayleigh-Plesset dynamics used by Nagalingam et al. [29] to analyse their findings. These observations suggest that the qualitative physics of focused and unfocused laser NPLIN may be the same.

This chapter's authors however do not wish to imply herewith that the IH mechanism is a full description of NPLIN. It currently fails to address exhaustively why some systems reportedly do not undergo NPLIN. It also does not answer completely whether there is any polarisation dependence in NPLIN, and whether sometimes there is polymorph control. See Table 1 for a summary of discussed mechanisms and respective shortcomings. These problems could be addressed by further research. The question of NPLIN susceptibility may hinge on a competition of diffusion and solubility increases with temperature on the one side and solute build-up due to evaporation on the other. The polarisation dependence may have something to do with the sensitivity of impurity particles towards laser light of varying ellipticity. Polymorph nucleation bias may or may not have to do with elevated supersaturations and temperatures. At present, these are only speculations and they are not discussed any further.

2.4 Protein NPLIN

Most NPLIN observations to date have involved small organic molecules such as urea, and existing theoretical models are primarily tailored to these systems. Proteins, however, are orders of magnitude larger and structurally more complex, and may not follow the same nucleation mechanisms. Furthermore, protein solutions typically contain extra components compared to small-molecule NPLIN experiments such as precipitants [38], buffer compounds, and other additives.[3] Finally, the few available protein studies employ femtosecond or picosecond pulsed lasers focused using objective lenses, producing intensities several orders of magnitude higher than those used in small-molecule work. Such conditions can even induce optical breakdown of the solvent. Altogether, these differences—in solution composition, solute molecular structure, and in laser conditions—may further influence the physics of NPLIN.

See Fig. 3 showcasing Adachi et al. [12]'s setup, which was the first study to report NPLIN of Hen Egg-White Lysozyme (HEWL). In this setup, a femtosecond laser beam is focused into a small container where protein droplets are isolated in either sitting or floating configuration. The studies mentioned in the rest of this section all use a variation of this setup. Yoshikawa et al. [39] and Iefuji et al. [14] used similar focused laser setups with femtosecond pulsed lasers with protein solutions instead of suspended droplets, together with simultaneous high-speed camera recording. Lee et al. [23] used unfocused picosecond and nanosecond pulsed lasers on hanging and suspended protein droplets.

NPLIN on HEWL has been performed by Lee et al. [23] using different laser conditions. Specifically, they used both picosecond (532 nm) and nanosecond (532 and 1064 nm) pulse lasers. 532 nm wavelength reportedly resulted in higher

[3] As discussed in chapter "The Starting Point for Biomolecular Crystallisation" of this book.

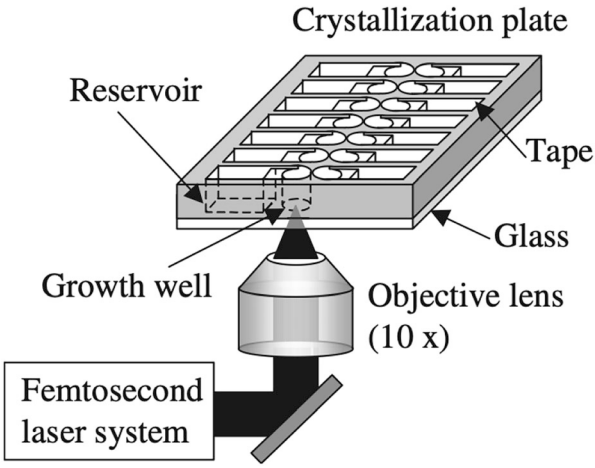


Fig. 3 Laser-induced nucleation setup used by Adachi et al. [12] in their experiments on Hen Egg-White Lysozyme (HEWL), featuring a focused femtosecond pulsed laser and micro-litre sample stages for sitting and floating protein drop experiments

NPLIN probabilities (55.1%) than 1064 nm (20.0%). They also reported that NPLIN probability decreased upon aging (for a picosecond 532-nm laser, 90% crystallised with 60-min aging time, whereas 3 hours resulted in a crystallisation probability of 13.3%), which could be due to diffusion diluting the proteins.

The impurity heating mechanism demands that the temperature of the solution locally reaches boiling point levels or higher. For aqueous systems, the resulting temperatures are high enough to cause degradation of proteins. Adachi et al. [12] reported degradation of irradiated HEWL samples only for higher laser pulsing frequency, keeping energy per pulse and wavelength constant. In their paper, they conjectured that it is the excess number of laser pulses that leads to degradation. Based on their observations, and considering the impurity heating mechanism, it could be that, for a single laser pulse, the degradation time at the vapour-liquid interface is longer than the nucleation time, but if many pulses are given, the entire liquid well is heated for a sufficient amount and time to cause degradation. In a later study by Yoshikawa et al. [39] on irradiating HEWL samples with 67 pulses from a femtosecond 780-nm laser, increasing pulse energy was first observed to increase nucleation probability, and then decrease, indicating an optimal laser energy for NPLIN on HEWL. Here too, it is possible that excess laser energy elevates the local temperature beyond the degradation point of the protein. At this point, this is only conjecture.

Yoshikawa et al. [39] observed the existence of laser threshold laser energy per pulse for HEWL below which no NPLIN was observed, and this threshold was very similar to the one they observed for cavitation to occur. This is in line with experiments by Nagalingam et al. [29] on KCl, suggesting the mechanism for NPLIN is very similar. In a later study by Iejufi et al. [14], they observed cavitation events proximal to glucose isomerase crystal formation. Furthermore, using cytochrome c which has a red colour, they observed regions of varying colour surrounding the cavitation event's collapse (see Fig. 4), with the colour variance being stronger with higher concentrations of cytochrome c. They conjectured that the

changing colours indicated increase of concentration of cytochrome c in the cavitation area, which would support the impurity heating theory. As with Nagalingam et al. [29]'s study, the laser was focused in this study, which could lead to sufficiently high laser intensities to cause optical breakdown-induced cavitation.

Whereas for small molecules NPLIN has been observed with nanosecond pulsed lasers, reports indicate that proteins require more even more concentrated laser pulses. Adachi et al. [12] could not crystallise HEWL with 23-ns laser pulses, but could do so with 120 fs laser pulses. In fact, most of the studies mentioned here involved femtosecond to picosecond lasers. An initial hypothesis would be that these lasers happen to irradiate with more energy; however, in the experiments by Lee et al. [23] on HEWL, a 100-ps laser was observed to result in significantly higher nucleation probability than a 5-ns laser, even though the total projected energy was 20 times lower (118–134 vs. 2209 mJ). The peak intensity of the picosecond laser was roughly a factor two higher (0.223–0.257 vs. 0.125 GW/cm^2), though. A conjecture is that even for similar projected energies, the absorption of light energy is stronger at higher beam intensities to photoionisation effects.

The studies mentioned largely seem to indicate that NPLIN on proteins depends on the same physics that are currently being used for regular NPLIN: Solution boiling leads to increased concentration of protein material, thus increasing supersaturation and nucleation rate. However, to what extent exactly aging time and degradation have to be considered, and whether NPLIN can be performed on all protein compounds in principle, remains unknown. This forms a basis for further research on protein NPLIN.

While most studies have addressed fundamental aspects of protein NPLIN, there has also been work in a more application-oriented context. Yennawar et al. [13] reported sizeable improvements in crystal quality, size, growth speed, and diffraction resolution for several proteins, including hen egg-white lysozyme, bovine pancreas ribonuclease B, sheep liver sorbitol dehydrogenase, Gluconobacter fructose dehydrogenase, and B. megaterium glucose dehydrogenase. To the authors' knowledge, however, this remains the only study of its kind, and additional work is required to assess the broader utility of NPLIN for protein crystallisation.

Taken together, current research suggests that protein NPLIN shares some mechanistic features with small-molecule systems, while also presenting additional challenges linked to protein stability. The single application-focused report hints at potential practical benefits, but the field remains too underexplored to draw firm conclusions. Overall, protein NPLIN stands as a promising yet largely open area of investigation, with large fundamental and application-based questions requiring further research.

2.5 Design Considerations

This short section aims to assist researchers new to the NPLIN field in designing corresponding basic experiments.

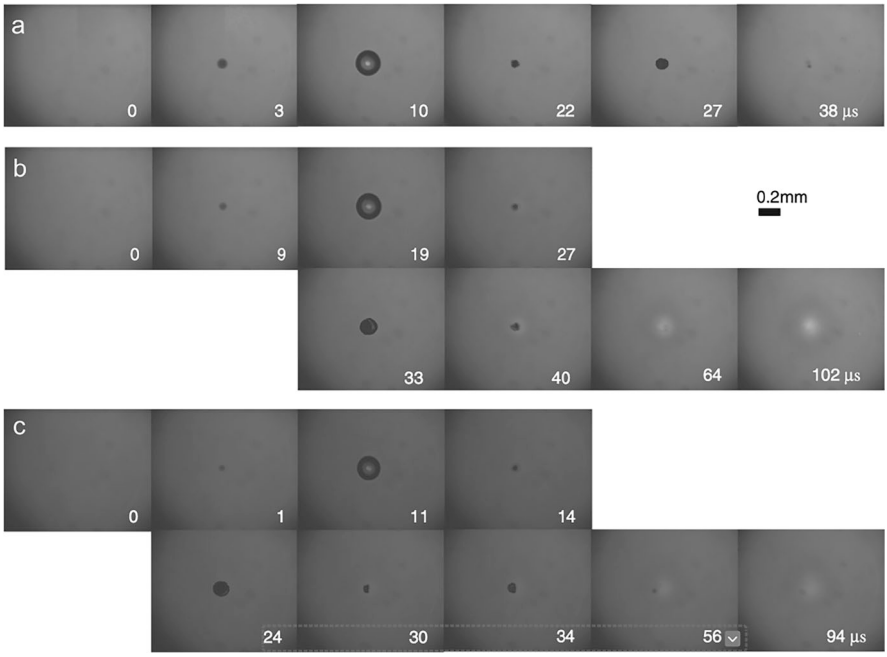


Fig. 4 Microscope recordings of cavitation under varying concentrations of cytochrome, reprinted from Iefuji et al. [14]. (**a**) In water. (**b**) 2 mg/ml cytochrome. (**c**) 10 mg/ml cytochrome. Note the brighter and darker regions surrounding the laser focus area after cavitation, which may be indicative of cytochrome concentration gradients due to cavitation

The first consideration when designing an NPLIN experiment is the specification of the laser, which will determine the appropriate choice of optical components such as mirrors and lenses. Korede et al. [6] summarised the laser intensities used across a broad range of NPLIN studies. For small molecules, most experiments operate in the range of 1–100 MW/cm^2. For proteins, significantly higher intensities—often several orders of magnitude greater—may be required. Focusing the laser using an objective lens can help achieve the necessary peak intensities. Commonly used laser wavelengths fall within the visible to near-infrared spectrum.

Once the laser has been selected, the next step is to establish the overall experimental setup. It is strongly recommended to place the laser, all optical elements, and the sample on a vibration-isolated surface such as an optical table. This enhances both precision and safety. Even minor vibrations can misalign optical elements or cause the laser to deviate from its intended path, posing risks to both equipment and personnel. Setup complexity can vary: from simple beam-on-vial configurations to advanced arrangements involving beam-splitting optics, high-speed cameras sharing the optical path, polarisation filters, and more.

Solution preparation is also a critical component of experimental design. Even with simple solutes such as KCl, complete dissolution of all solute materials prior

to irradiation is essential for reproducibility and interpretation. Other factors such as solution aging time and the timing of antisolvent addition (where applicable) are not yet fully understood and might affect crystallisation outcomes. Therefore, rigorous documentation and consistency in sample preparation protocols are crucial.

In many cases, crystal formation does not occur immediately after laser irradiation. Samples may require a rest period before nucleated crystals become visible. During this time, it is important to maintain stable conditions—particularly temperature—to avoid influencing nucleation post-irradiation. For example, placing irradiated vial samples back into a temperature-controlled bath can help maintain environmental stability during this resting phase.

3 Other Field Control Techniques

3.1 Other Light Irradiation-Based Techniques

In contrast to the femtosecond-to-nanosecond pulsed laser-based method described in the previous section of NPLIN, longer duration (seconds to hours) continuous-wave lasers can also induce nucleation. One prominent example is laser optical trapping: The electric-field gradient created by a focused laser beam forms a potential well that concentrates solute molecules at the beam centre, facilitating crystal nucleation [6]. For instance, Yuyama et al. [40] demonstrated controlled, local crystallisation of lysozyme this way.

It is also possible to induce nucleation photochemically. See for instance the technique discussed by Okutsu [41] on lysozyme. Here, it is believed that the π electrons in Trp, Tyr, and Phe amino acids are activated leading to the formation of protein dimers, and that these dimers act as a template for the formation of crystal nuclei.

3.2 Electric Fields

In 1972, Kaschiev [42] estimated the impact of an electric field on the nucleation work. Depending on the permittivities of the solid and liquid phases, the formation of a nucleus might be made more or less favourable in the vicinity of an electric field.

This work term however is only one of several phenomena caused by the presence of charged electrodes. Taleb et al. [43] found, in their study on crystallising lysozyme, that the application of an electric field seemed to induce a concentration gradient surrounding the electrodes. In a review paper by Hammadi et al. [7], they discuss these effects, and others including potential electrochemical reactions, in more detail.

3.3 Magnetic Fields

Based on a fairly old review article by Yin [5], the effects of a strong magnetic field on crystal nucleation are not yet fully understood. Several of the discussed articles [2, 44, 45] seemingly report a decrease in nucleation rate under the presence of a magnetic field, but a mentioned study by Gavira et al. [46] reports enhancement of nucleation rate. The exact mechanism is not discussed in detail, although Yin [5] does mention that magnetic fields can affect natural convection in solution, viscosity, and diffusivity, which may all affect apparent nucleation rate.

3.4 Ultrasound

Nucleation introduced by ultrasonic (> 20 kHz) acoustic waves, commonly known as sonocrystallisation when applied to crystallisation processes, is a well-established field with a long history. A detailed, modern overview is provided by Jordens et al. [8], discussing key observations, mechanistic theories, and practical guidelines for sonocrystallisation of chemical and in particular pharmaceutical systems.

Although the effects of ultrasound on nucleation via induction time reduction are well-documented, the underlying theoretical mechanism remains unclear. To the authors, it seems like prevailing models converge on the importance of acoustic cavitation [47], in which high-intensity ultrasound induces the formation, growth, and collapse of vapour bubbles in the liquid phase. These events may generate localised zones of high pressure and temperature, intense shear forces, and shockwaves, all of which may facilitate nucleation but also post-nucleation phenomena such as aggregation and breakage.

Interestingly, this centrality of cavitation events parallels the impurity heating hypothesis in NPLIN, and might form the basis for new research questions. One may ask questions such as whether the bubbles formed in sonocrystallisation are of similar size and lifetimes, and undergo similar dynamics, as those observed in NPLIN experiments.

4 Conclusion and Outlook

This chapter reviewed various techniques to control biomolecular crystal nucleation via external fields, with a primary focus on non-photochemical laser-induced nucleation (NPLIN). See Table 2 for a summary of these techniques.

Conventional NPLIN involves the appearance of crystals in supersaturated solutions following pulsed laser irradiation. While most commonly applied to solution crystallisation, it has also been demonstrated for dissolved gases [16, 17] and single-component systems [18, 19]. Key experimental observations include the existence of

Table 2 Summary of external field-based biomolecule crystal nucleation techniques

External field	Presumed effect on nucleation
High-energy pulsed lasers (NPLIN)	Instant nucleation
Continuous-wave lasers (optical trapping)	Enhanced nucleation
Electric field	Reduced or increased nucleation rate (depends)
Magnetic field	Reduced nucleation rate
Ultrasound	Increased nucleation rate

a threshold laser intensity, the strong influence of impurities or nanoparticles suspended in solution, and the broad applicability of the technique across different compounds. Current understanding suggests that NPLIN involves laser-induced heating and solvent boiling, although the precise link between these physical events and crystal nucleation remains under debate.

NPLIN has been successfully applied to a range of biomolecules, including amino acids such as glycine [18] and proteins such as lysozyme [12, 23, 39]. The limited number of studies involving protein NPLIN suggests that the underlying mechanism is very similar to that of small-molecule NPLIN. This in turn suggests that findings in small-molecule NPLIN may likely be used as clues for protein NPLIN and vice versa. Several important questions specific to biomolecular crystallisation remain unresolved:

1. Is NPLIN universally applicable to biomolecular crystallisation, or are there limits?
2. Does NPLIN promote selective crystallisation of certain polymorphs?
3. Under what conditions might laser exposure lead to protein degradation?
4. What is the influence of aging time prior to irradiation?

Beyond NPLIN, this chapter has also discussed several other external-field-based nucleation techniques:

1. Optical trapping, where an electric field gradient concentrates solute at the laser focus
2. Photochemical reactions leading to nucleation
3. Electric fields, which can affect both solute concentration gradients and nucleus stability
4. Magnetic fields, which in some cases appear to inhibit nucleation
5. Ultrasound (sonocrystallisation), which is reported to accelerate nucleation, potentially through cavitation

While caution is warranted in making generalisations, common patterns emerge across these techniques. In each case, the external field appears to influence one or more of the following:

1. Solute concentration gradients
2. Thermodynamic stability of solute material critical nuclei
3. The kinetic nucleation prefactor, potentially through altered solute mobility

These parallels suggest that deeper comparisons between methods may yield valuable insight. For example, it is worth asking whether cavitation events in sonocrystallisation and thermocavitation in NPLIN share similar bubble dynamics—such as size and collapse time—and whether any potential differences observed can be linked to differences in nucleation behaviour. Systematic investigations along these lines could help clarify the fundamental mechanisms at play and uncover whether deeper, possibly unifying, physical bases connect these diverse nucleation phenomena.

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