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Novel Demonstrations of Optical Refrigeration in Enzymes and Polystyrene and TEM Imaging of Novel Fluoride Microstructures

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Abstract

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This work focuses on novel demonstrations of optical cooling in structures that have not been cooled via laser refrigeration previously. The primary study was focused on investigating the possibility of optical refrigeration being powerful enough to cool enzymes attached to the surface of the cooling crystals, with potential applications in biochemical techniques requiring thermocycling, such as the polymerase chain reaction. Another study focused on the potential development of radiation balanced whispering gallery mode lasers through use of cooling nanoparticles deposited on the surface is also presented. Finally, transmission electron microscopy work that helped to uncover novel microstructures in common rare earth-doped fluoride materials that would not have been discovered without use of the instrument.

Chapter 1 presents an introduction to the concepts of optical refrigeration and the thermometry techniques used to track the temperatures of our optical refrigeration materials. The parameters that must be carefully balanced to attain optimal laser refrigeration are introduced and discussed in detail. The two primary methods of thermometry and the concepts that underlie them are also presented.

Chapter 2 introduces the idea of optical refrigeration of enzymes by describing the tech-

nique we hope it may eventually be applied to, that being the polymerase chain reaction. Data is presented showing the development of a synthetic technique for covalently attaching a test enzyme, horseradish peroxidase, to the surface of the cooling ytterbium-doped yttrium lithium fluoride crystals. It is shown that the enzymes remain functional while attached to the crystals and that the enzymatic reaction product is not generated by any source other than the bound enzymes. A data collection apparatus and method are designed in order to obtain comparable enzymatic reaction rate data across heating, cooling, and control conditions from a single confirmed cooling crystal coated with the enzymes, and experiments using this setup and method can repeatedly show reaction rate increase during heating trials, reaction rate decrease during cooling trials, and control trials landing in between them. This is the first demonstration of optical refrigeration of enzymes.

Chapter 3 involves cooling experiments using polystyrene resonators coated with ytterbium-doped upconverting nanoparticles with the goal of creating a radiation balanced microlaser as well as provide an improved substrate for single molecule biophysics. Multiple thermometry methods are used to probe the temperatures reached at the surface of the microspheres and their surrounding environment. It is found that local cooling of the crystals at the surface of the crystals is possible, but overall cooling of the local environment is not achieved. However, the coated spheres demonstrate significantly reduced heating when compared to identical uncoated spheres of the same diameter.

Chapter 4 largely departs from the subjects of the rest of this dissertation and moves to electron microscopy of novel structures synthesized within the Pauzauskie group. The two structures presented are of porous α phase sodium yttrium fluoride crystals and core-shell calcium fluoride crystals referred to as mandala structures. Data generated from multiple

advanced techniques are presented, including elemental maps from energy-dispersive x-ray spectroscopy and three dimensional tomographic reconstructions. Hypotheses describing the possible reasons for growth of these unusual structures are presented and potential future applications for these crystals are discussed.

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GLOSSARY

CBM: Cold Brownian motion

DNA: Deoxyribonucleic acid

EDS: Energy-dispersive x-ray spectroscopy

HRP: Horseradish peroxidase

HRPYLF: YLF crystals with HRP enzymes bound to their surfaces

NAYF: Sodium yttrium fluoride, written as NaYF to denote its nonstoichiometric nature

NIR: Near infrared

PCR: Polymerase chain reaction

PSD: Power spectral density

QPD: Quadrant photodiode

SEM: Scanning electron microscopy/microscope

STEM: Scanning transmission electron microscopy/microscope

TEM: Transmission electron microscopy/microscope

UCNP: Upconverting nanoparticle

UV-VIS: Ultraviolet-visible range of light

WGM: Whispering gallery modes

YLF: Yttrium lithium fluoride, YLiF_4

Some passages have been quoted or adapted verbatim from the following sources:

- **R. Gariepy**, A. J. Nuhaily, Z. Feng, and P. J. Pauzauskie. “Optical Refrigeration of Enzymes Attached to Rare Earth-doped Fluorides” (*in preparation* 2025).
- X. Xia*, **R. Gariepy***, R. G. Felsted, A. Teitelbom, E. Chan, and P. J. Pauzauskie. “Solid-state laser refrigeration of core-shell polystyrene microspheres” (*in preparation* 2025).
- L. Forberger, **R. Gariepy**, A. B. Bard, M. A. Hilyard, Z. Feng, S. Hirani, and P. J. Pauzauskie. “Porous α -NaYF – A Pressure Dependent Laser Refrigeration and Heating Material” (*in preparation* 2025).

* *Indicates equal contribution*

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DEDICATION

to Nick, my love

and Juno, my spirit

Chapter 1

INTRODUCTION

Optical refrigeration is a phenomenon that, at first glance, seems as though it should not be possible. In fact, after it was first proposed by Pringsheim in 1929 in vaporized sodium atoms, concerns about this process defying the second law of thermodynamics abounded.[1] Some years later, Landau more closely examined the entropy of an optically refrigerated system and determined that the system aligns with the second law so long as the input light source has very low entropy.[2] Even with this strong theoretical backing, solid-state laser cooling was not demonstrated experimentally until 1995, as the discovery of the laser was a necessary prerequisite for optical cooling to be possible.[3] In the time since then, a great deal of progress has been made in this field, from the Doppler cooling of vapors that Pringsheim first proposed,[4] to solid-state cooling down to cryogenic temperatures,[5, 6, 7, 8] to optical cooling in semiconductors using rare earth fluoride crystals,[9], to cooling in aqueous media.[10]

Work in the Pauzauskie lab typically focuses on crystals in the micron-scale range, which come with their own advantages and challenges compared to working in the bulk regime. Smaller crystals are less likely to reabsorb photons emitted from the laser cooling dopants due to their size, reducing net heating from nonradiative pathways. However, their small size also means significantly lower laser absorption coefficients due to reduced ytterbium number density compared to large-scale crystals.[11, 12] The exact factors that quantitatively describe this balancing act will be discussed in section 1.1. There are also the standard difficulties of

working with smaller scale materials, such as achieving consistent synthesis results (which is especially important for optical cooling materials), the need to isolate single crystals for measurements, and designing and accurately carrying out practical measurement methods.

The focus of this dissertation will be on solid-state cooling in ytterbium-doped fluorides and its applications in the realms of biophysics and radiation-balanced microlasers. The primary results presented demonstrate novel advancements in what types of materials can be effectively optically cooled and how those results can be applied to such techniques as PCR, single-molecule or single-protein optical trapping experiments, and lasing. Additional novel ytterbium-doped fluoride structures discovered through TEM imaging, elemental analysis, and tomography are also presented as possible future candidates for interfacing with biological molecules for optical cooling applications.

1.1 Optical Refrigeration

As briefly mentioned in the previous section, the invention of the laser was key to the phenomenon of solid-state optical cooling to be realized. This is because lasers have incredibly low entropy compared to other forms of light. The stimulated emission created within a laser produces near identical photons, creating a beam with low divergence, high spatial and temporal coherence, a narrow range of wavelengths, and well-defined polarization. Comparatively, the spontaneously emitted photons produced during the solid-state cooling process will have a significantly higher entropy due to having no defined direction, phase, or wavelength. This means that we can all rest easy knowing that this process does follow the known natural laws and the second law of thermodynamics remains intact.

Unlike the Doppler method used for the cooling of gases, solid-state optical refrigeration occurs through a process known as anti-Stokes luminescence. In traditional Stokes luminescence, photons with an energy lower than that of the pump photon are emitted and the

energy difference between these two photons is dispersed as heat within the luminescent material's crystal lattice, thus heating the system. Conversely, anti-Stokes luminescence results in a photon of higher energy than the pump photon being emitted from the luminescent material, requiring energy to be removed from the crystal lattice in the form of phonon energy in order to balance the process.[13] A simplified four-level diagram that describes the anti-Stokes luminescence process is shown in Figure 1.1a.[11]

This four-level example describes a system with a ground state manifold and an excited state manifold split into two levels. These levels can be effectively thought of as the Stark levels generated in the ytterbium atom due to the crystal field of the surrounding crystal lattice. The actual crystal field splitting of ytterbium is shown in Figure 1.1b, demonstrating that there are more levels present in actuality than are shown in the example, but the process is the same. Firstly, a photon of a known, carefully chosen energy $h\nu$ will promote an electron from state $|1\rangle$ to state $|2\rangle$. This movement disrupts the distribution of states within each manifold, which is based on the thermal energy of the system and follows a Boltzmann distribution. As a result, thermal energy from the lattice is consumed in the form of phonons to reestablish equilibrium at a rate of w_g for the ground state manifold and w_e for the excited state manifold. Spontaneous emission then occurs, releasing a photon of higher energy ($h\nu_f$) than what was originally absorbed. These various radiative relaxation pathways occur at a rate W_r , while nonradiative relaxation pathways caused by multiphonon relaxation or photon reabsorption occur at a rate W_{nr} . It is assumed that the previously mentioned thermalization processes take place at significantly shorter time scales than the ensuing photon emission. In the case of Yb^{3+} ions, their excited state lifetimes are on the order of milliseconds due to spin-orbit coupling, much longer than the time scale needed for thermalization to take place.[14] In large enough quantities, this will eventually lead to

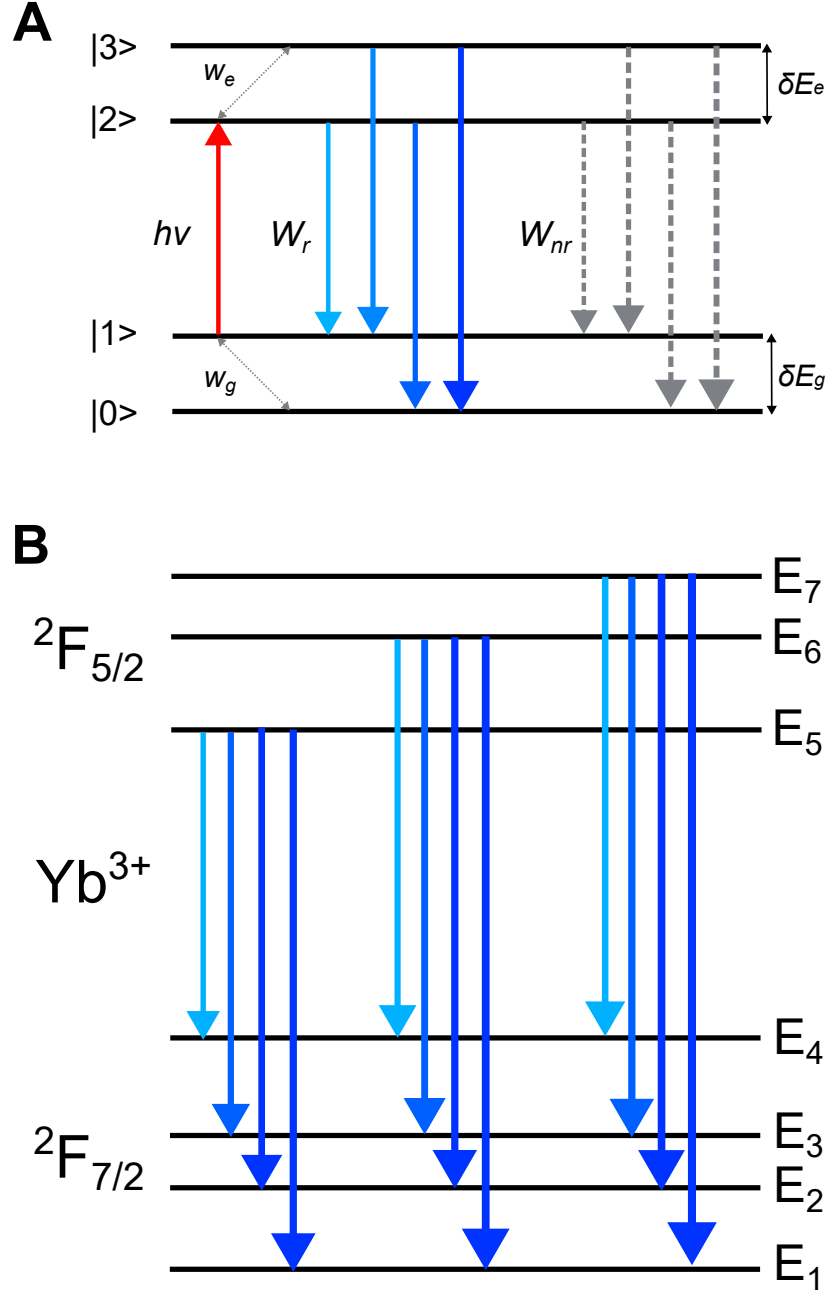


Figure 1.1: (A) The simplified four level model for describing optical refrigeration consisting of a ground state manifold with levels $|0\rangle$ and $|1\rangle$ and excited state manifold with levels $|2\rangle$ and $|3\rangle$, as described in reference 11. (B) The actual energy levels of Yb^{3+} ions showing the four-level lower manifold and three-level upper manifold.

laser refrigeration of the system, assuming that the host material demonstrates the beneficial properties that we will now discuss.

There are a few key parameters that a system should demonstrate in order to be a good candidate for laser cooling. These three parameters are cooling efficiency, absorption efficiency, and external quantum efficiency. Firstly, the cooling efficiency of an ideal system is given by the equation:

$$\eta_c = \frac{h\nu_f - h\nu}{h\nu} \approx \frac{k_b T}{E_g} \quad (1.1)$$

in which k_b is the Boltzmann constant, T is the temperature, and E_g is the energy of the ytterbium ion's lowest ground state.[11] As can be seen from this equation, a large anti-Stokes shift is advantageous for attaining a sizable cooling efficiency. This would suggest that a large crystal field splitting would be a desirable trait, as the larger energy gaps between levels would require more phonons from the lattice to thermalize the manifolds, leading to more thermal energy being removed from the lattice. This is, like many things, only partially true and brings me to our next relevant parameter: the absorption efficiency.

The absorption efficiency of a given crystal tells us the proportion of inputted laser photons that are absorbed by a cooling ion and is given by the equation:

$$\eta_{abs} = \frac{\alpha(\lambda, T)}{\alpha(\lambda, T) + \alpha_b} \quad (1.2)$$

where $\alpha(\lambda, T)$ is the wavelength- and temperature-dependent absorption coefficient of our cooling ion and α_b is the background absorption coefficient, which is enhanced by the presence of impurities in the crystal lattice. Equation 1.2 clearly shows the importance of a carefully synthesized sample with minimal impurities, which is a much easier property to control via synthesis conditions than the resonant absorption coefficient. Speaking of, this resonant absorption coefficient can be defined as:

$$\alpha_r = \sigma_{12} N_t \frac{e^{-\delta E_g / k_b T}}{1 + e^{-\delta E_g / k_b T}} \quad (1.3)$$

where σ_{12} is the absorption cross-section of our resonant $|1\rangle$ to $|2\rangle$ transition, N_t is the total density of ions in our system across all four states, and δE_g is the energy width of our ground state manifold. This equation elucidates further the balancing act that we must strike between the factors that improve cooling efficiency and those that improve absorption efficiency in order to achieve maximum optical cooling. As the width of our ground state manifold increases, the exponential term will begin to converge towards zero. Additionally, as the solid is cooled, the $k_b T$ term will also shrink and push the absorption coefficient even lower. Physically, as thermal energy in our system is removed, electrons are no longer capable of thermalizing up to the highest energy ground state level in order to undergo the resonant transition. With no photon absorption, there can be no cooling. As such, it is important to find a good middle ground between a crystal field splitting that allows for a sizable anti-Stokes shift while not being so large that only slight cooling prevents thermalization, and thus photon absorption, from occurring.

The final parameter that should be discussed is the external quantum efficiency of the solid. This is a fairly similar parameter to the absorption efficiency, but describes the followup phenomenon to absorption: emission. It is described by the following equation:

$$\eta_{ext} = \eta_e \eta_i = \eta_e \frac{W_r}{W_r + W_{nr}} \quad (1.4)$$

where η_e is the photon escape efficiency and η_i is the internal quantum efficiency, also defined in terms of the rates of radiative and nonradiative relaxation. The internal quantum efficiency tells us the rate at which absorbed photons are emitted via anti-Stokes fluorescence and the escape efficiency tells us if the photon makes it out of the solid without being trapped via refraction or reabsorption. Ideally, we want both of these values to be as close to unity as possible for similar reasons as described previously. Any nonradiative relaxation pathways introduce heat to the system and any emitted photons that get trapped in the solid cannot

remove any heat from the system either.

Now that we have discussed the three major parameters we must keep in mind for optimal laser refrigeration in nonideal systems, we can go back to equation 1.1 and rewrite it to account for these parameters:

$$\eta_c = \eta_{abs}\eta_{ext}\frac{h\nu_f - h\nu}{h\nu} \quad (1.5)$$

As can be seen, if our absorption efficiency and our external quantum efficiency are near unity, we recover equation 1.1 from equation 1.5. As such, our goal is to keep these parameters as close to 1 as possible by selecting a crystal system for our ions that can be carefully synthesized to be highly pure and with beneficial crystal field splitting, phonon energies, and refractive index.

It was in the 1950s that it was first proposed that rare earth ions could be good options for practical optical refrigeration because the 4f orbital electrons are well shielded by higher quantum number orbitals.[11, 15] This reduces the likelihood of nonradiative relaxation, a boon for optical refrigeration. After the laser was invented and further developed in the sixties, the first attempt to realize laser cooling was made with middling results.[16] Kushida and Geusic observed reduced heating in their Nd³⁺:YAG crystal rather than the cooling they hoped for, but it was a start. It was the work of Richard Epstein and his colleagues at Los Alamos National Lab in 1995, nearly forty years later, who first demonstrated net laser cooling in a ytterbium-doped ZBLANP glass material.[3] After this point, a wide number of possible laser cooling hosts for ytterbium were discovered, including additional glasses,[17, 18, 19, 20] oxides,[21, 22, 23], and of course, fluorides.[5, 6, 7] Fluoride crystals are particularly effective optical refrigeration hosts due to their relatively low refractive indices at the target wavelengths and low phonon energies, making multiphonon relaxation significantly less likely and maintaining a very high quantum efficiency as a result.

Other rare earth atoms, such as thulium, erbium, and holmium, have also shown refrigeration but it is 10% ytterbium-doped YLF that currently demonstrates the greatest cooling potential, having reached cryogenic temperatures as low as 91 Kelvin.[8, 24, 25, 26, 27] This shows that YLF is the current best known practical host for attaining the greatest optical refrigeration, with its crystal field splitting hitting a strong balance between anti-Stokes shift and absorption efficiency. Another key property, especially for the enzyme work in Chapter 2, is that hydrothermally grown Yb:YLF microcrystals are capable of net cooling even in aqueous environments.[10] This was an important contribution to optical refrigeration studies considering most previous optical cooling work is done in vacuum, as aqueous environments present challenges for cooling due to water's high heat capacity and absorption in the infrared range where laser cooling takes place in Yb:YLF.

Another fluoride of interest for this report is sodium yttrium fluoride, or NaYF. When looking purely at estimated crystal field splitting energies, it is thought that NaYF would actually be a more effective optical refrigeration lattice than YLF.[28] However, in practice, NaYF is often a non-stoichiometric compound and so its exact crystal field splitting cannot be well understood or predicted.[29, 30] As a result, its theoretical cooling potential has not yet been maximized, though cooling results have been shown, including by the Pauzauskie group.[31] NaYF has found its primary home in non-optical refrigeration related upconversion applications due to its status as the most efficient known upconversion material when doped with Yb^{3+} or Er^{3+} . [32] This particular fluoride is used for the cooling of polystyrene microresonators in Chapter 3, and will receive a more in-depth introduction in that chapter.

1.2 Thermometry Methods

Considering the primary concern of this dissertation is the demonstration of optical cooling, especially materials that would heat without the influence of rare earth-doped fluorides, it is

important that we understand the thermometry methods used to confirm our cooling results. All of these methods are noncontact and make use of temperature's observable effect on either the distribution of states within the crystal itself or on the viscosity of the solvent the work is being done in (typically water). In addition to being necessary due to thermocouplers not being available in "nano" size, noncontact methods remove the possibility of a conductive heat flux generated by any electronic measurement implement physically contacting our sample.

These measurements are taken on a home built optical trapping setup that is a modified optical tweezer kit from ThorLabs. A diagram showing the optical layout of the table is shown in Figure 1.2.

1.2.1 Mean Fluorescence Thermometry

The primary method of temperature measurement used throughout these projects is mean fluorescence thermometry. This method can measure temperature change through the photons released by the Yb^{3+} ions in the fluoride lattice. As previously described, thermal energy from the lattice is used to reestablish the Boltzmann distribution of states during the laser cooling process. However, as the temperature of the lattice changes, the exact distribution of states changes as well, which shifts the emission peaks slightly and changes the mean fluorescence wavelength. If we see this value blueshift with increasing laser power, this means that the crystal is heating, whereas a redshift would indicate cooling. This mean fluorescence wavelength is calculated using the following equation:

$$\lambda_f = \frac{\sum \lambda_i I(\lambda_i)}{\sum I(\lambda_i)} \quad (1.6)$$

in which λ_i is the wavelength at a given pixel i and $I(\lambda_i)$ is the emission intensity at that given wavelength. The summation takes place over the entire emission wavelength range of

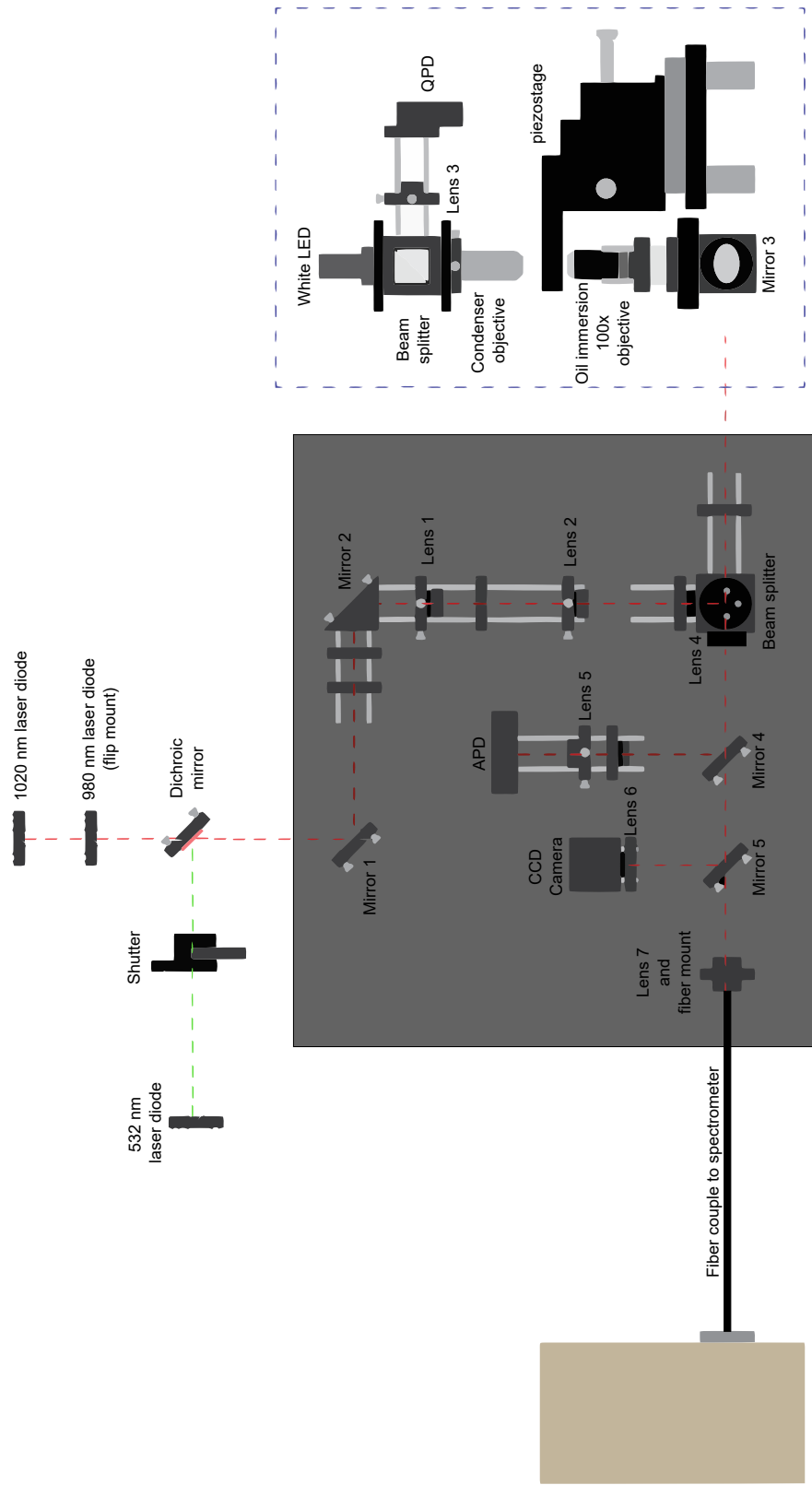


Figure 1.2: Diagram of the optics table used to perform the experiments described in this manuscript. Diagram shows the laser sources, elevated optics section with beam expansion region and data collection instruments, vertical microscopy setup, and path to spectrometer for data collection.

the spectrum and integrates the area underneath the spectrum, which is especially useful for host lattices like NaYF which do not have enough well-defined and separated peaks. It also makes the technique less susceptible errors generated when having to select specific areas of the spectrum.

1.2.2 Cold Brownian Motion Thermometry

Mean fluorescence thermometry gives us a good understanding of the temperature effects happening within our crystal of interest, but what about its effect on the surrounding environment it finds itself in? Cold Brownian motion thermometry can help answer this question. This technique allows us to track the random motion of a particle held within an optical trap, correlate it with the temperature dependent viscosity of the surrounding solvent, and extract a diffusion coefficient from that data. Using this technique, the Pauzauskie group was able to demonstrate the first instance of cold Brownian motion since Einstein's seminal paper in 1905.[10, 33]

Once the particle of interest is stably trapped by the input laser, the light scattered by the particle is collected by a condenser objective and sent to a quadrant photodiode (QPD). The piezostage the sample is mounted upon is also oscillated at a known amplitude and frequency, typically 32 Hz, for calibration purposes. The QPD can track the random motion of the particle in the trap, which can be converted via Fourier transform to a power spectral density (PSD) signal. This PSD can be fitted to the following equation:

$$P_T(f) = \frac{D}{\pi^2(f^2 + f_c^2)} \quad (1.7)$$

where f is the oscillation frequency of the piezostage, in order to derive the temperature dependent diffusion coefficient D in units of V^2/s and the corner frequency f_c . The oscillation of the piezostage also introduces a delta function spike into the spectrum, which allows us

to derive the calibration constant necessary to convert the diffusion coefficient from units of V^2/s to the more standard m^2/s . From this, the temperature of the crystal's surroundings can be derived.[34]

Chapter 2

OPTICAL HEATING AND REFRIGERATION OF ENZYMES USING RARE EARTH DOPED FLUORIDES

2.1 Introduction

2.1.1 Polymerase Chain Reaction

The polymerase chain reaction, or PCR, is one of the most invaluable and widely used techniques in fields such as biochemistry, genetics, and medicine. The method's relative simplicity combined with high efficacy and wide variety of applications has led to its meteoric rise in popularity since it was first discovered by Randall Saiki et al. in 1988.[35] PCR was originally designed to amplify a target DNA strand to make it more detectable by future analytical methods, such as enzyme-linked immunosorbent assays or gel electrophoresis, for uses such as direct genomic sequencing and identification of mutations.[36, 37, 38] Today, PCR is still used for those same applications, as well as forensic science, paternity testing, and infectious pathogen detection, including detection of COVID-19.[39, 40, 41, 42]

A PCR sample is made up of a complex mixture of biological molecules and ions in a pH 8.3 buffer. The most important aspects of the solution are the template DNA to be amplified, an enzyme known as DNA polymerase, and oligomeric forward and reverse RNA primers, which tell the enzyme where to begin building the copied DNA.[43] The polymerase is the key to PCR's success, as it is what builds the strands of copied DNA out of free nucleotides formed in solution. Every organism that has DNA has its own unique form of DNA polymerase that aids in DNA synthesis within its body, but not every polymerase has

the same properties. For example, human DNA polymerase would be far too temperature sensitive to be used in PCR due to the high temperatures reached during PCR thermocycling, causing it to denature and stop functioning. To counteract this, the technique makes use of a specific, thermally stable DNA polymerase isolated from a bacterium found near underwater thermal vents called *Thermus aquaticus* (often abbreviated to *Taq*) which can withstand much higher temperatures than our own enzyme.[44, 45, 46] The final critical portion of the PCR matrix is Mg^{2+} ions, added in the form of $MgCl_2$ salt. The magnesium ions catalyze the reaction significantly, reducing average experiment time from several hours to roughly one hour.[47]

PCR takes place in a series of multi-step cycles, which occur at different temperatures and for different lengths of time. A simplified schematic of a single PCR cycle is shown in Figure 2.1. The first step in a PCR cycle is known as denaturation. During this step, the double-stranded DNA is split into two single strands, one in the 5'-3' direction and one in the 3'-5' direction, to be built upon in later steps. The high temperature of 95 °C lyses the hydrogen bonds that hold together the nucleotide pairs. After the denaturation step, the annealing step takes place. This step takes place at a much lower temperature, which is dependent on the specific primers present in the PCR matrix. The primers are specifically designed to bind to the targeted DNA strands, directly before the portion of the DNA that will be copied. This designates the location for the *Taq* polymerase enzyme to begin generating the copied strand of DNA from the free nucleotides in solution. The polymerase does its work during the elongation step, which is the third and final step in a single PCR cycle. This step occurs at 72 °C, which is the optimal working temperature for the *Taq* polymerase enzyme to build the copied DNA. The time taken for this step is variable, dependent upon the number of nucleotide base pairs in the target DNA strand. The more base pairs in the

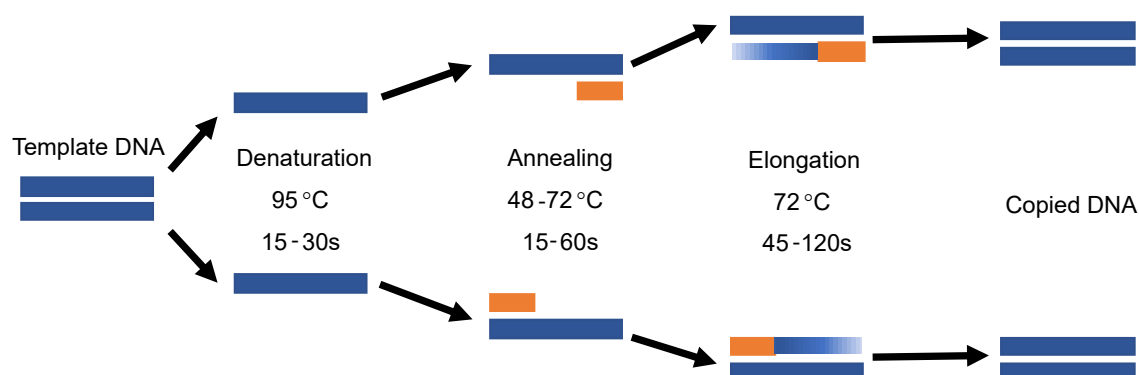


Figure 2.1: A simplified schematic of a single PCR cycle, including names, temperatures, and hold times for all three steps of PCR.

target, the longer the elongation step will take. A full PCR trial contains anywhere from 30 to 40 individual cycles, ideally leading to millions or billions of copies of the target DNA.[47]

A great deal of work has gone into developing variations of PCR, which allow the technique's utility to expand even beyond the copying of short portions of DNA. Quantitative PCR, or qPCR, is rapidly becoming the standard for PCR procedures because it allows for real time monitoring of the PCR product.[48] Fluorescent agents present in solution bind to the freshly copied DNA strands, increasing in fluorescence as more product is created in solution. This is highly useful for identifying if your trial is successful without the long wait time necessary for a full experiment. Reverse transcriptase PCR (RT-PCR) is another novel variation developed that is used to amplify RNA rather than DNA. This technique is highly useful in the field of pathogen detection and disease research, as some viruses, such as COVID-19, only contain RNA. Unlike DNA, RNA is single stranded, which causes problems when it comes to creating a direct copy of the RNA. Therefore, an additional enzyme known as a reverse transcriptase is utilized to create a complementary DNA strand that can be amplified by the polymerase for later detection, and its presence can be linked to the presence of the target RNA. Other method variations were created to amplify longer segments

of DNA by using multiple polymerases (long-range PCR) or multiple segments of a target DNA strand using multiple unique primers (multiplex PCR).[37, 49] A great deal of work has been done to observe the structure and function of *Taq* polymerase, as its full mechanism is not entirely understood.[50, 51, 52] Some work has also been dedicated to modification of the *Taq* polymerase enzyme for purposes such as inclusion of amino acids other than adenine, cytosine, guanine, and thymine, improved nucleotide addition and accuracy, and better binding to DNA in vitro.[50, 53, 54]

2.1.2 Motivation

Although there are many different PCR variations, one thing that they all have in common is the need for temperature cycling across the three steps of the process. The changes in temperature involved in PCR can be quite large, sometimes as large as 50 °C. Both the length and efficacy of the experiment are highly dependent upon how rapid and how accurate these temperature changes are. The length is most heavily impacted by the cooling step between the denaturation and annealing steps. It can take a great deal of time for water to cool naturally due to its high specific heat capacity, adding time to each individual cycle and compounding into the total experiment time.[47] Additionally, if the temperatures reached during PCR thermocycling are inaccurate by even one degree Celsius, the annealing and elongation steps are highly susceptible to failure.[55] In a typical PCR experiment, it is important that each cycle yields successful DNA copies, because without enough DNA yield, analytical techniques meant to detect the amplified DNA will not identify its presence.

The standard instrument used for PCR experiments is fittingly known as a thermocycler. These machines make use of external Peltier heating and cooling elements to heat up and cool down a high number of samples as they move between each step of PCR. Due to the external nature of the heating elements, that PCR samples are held within plastic tubes,

and that PCR samples are mostly water, complex heat transfer calculations are necessary to make certain that the temperature of the samples does not go too high or too low during the process. Although modern thermocyclers are slowly becoming better at this, they are also becoming more expensive, and any changes to the PCR sample or sample holder that conflict with the thermocycler's programming could cause failed experiments. Additionally, the sample tube will heat up from the outside in because of the external heaters, creating a temperature gradient across the samples. Having different parts of the samples achieve the target temperature at different times means that the hold times will not be consistent across the sample, leading to higher chances of failed experiments as well. The use of thermoelectric heating and cooling elements also pose stringent space and energy requirements, which makes traditional thermocyclers poor candidates for point of care applications, which are becoming increasingly important in a post-COVID world.

Therefore, a direct method of temperature control for PCR reactions could solve a number of issues present in the current method. Some researchers have developed a solid state method for thermocycling of PCR.[56] By using a nanoliter flow cell split into three sections, each held at a one of the temperatures for a step of PCR, and the sample solution is slowly pushed through the cell at a rate that keeps the solution in each section for the required amount of time, completing the reaction after 30-40 trips between the sections. Other previous work has shown promise in superparamagnetic iron nanoparticles dispersed in solution that heat up in an alternating magnetic field, though aggregation within the colloidal solution formed by the addition of these nanoparticles has proved to be troublesome for methods utilizing them.[57, 58] Other methods attempt to increase the efficiency and efficacy of PCR by introducing additional catalytic materials to the PCR matrix, such as gold nanoparticles and derivatives of tetramethylammonium chloride, without altering the thermocycling at

all.[59, 60, 61, 62]

Another field of high interest is what is known as “photonic PCR.” This method for PCR eschews the use of traditional thermocyclers in favor of using photonic nanoparticles dispersed within the sample matrix to uniformly heat the sample with an external light source, be it LED- or laser-based. This method has been of particular interest due to a few factors: its noncontact heating method, more efficient energy conversion and heat transfer, and the fact that the heating is volumetric rather than surface-based.[63] Photothermal heating of enzymes using NIR irradiation is also well characterized, including enzyme-metal nanoparticle composites. [64, 65, 66, 67] Gold nanorods are the most popular photonic nanoparticles used for these experiments due to gold’s efficient photothermal conversion, in addition to their other useful catalytic properties mentioned above. Multiple groups have made use of polyethylene glycol-functionalized gold nanorods and a 808 nm NIR laser heating source that demonstrate heating rates much higher than the traditional thermocycler, and one group even integrated a real-time quantitative feedback loop by taking advantage of DNA’s absorption of UV light from a diode.[68, 69] Other popular materials for these applications include graphene oxide nanoparticles integrated into a polymer matrix, through which the PCR solution is then flowed, and iron oxide nanoparticles, which function similarly to the gold nanorods in solution.[70, 71] All of these methods show strong progress in speeding up the heating steps necessary for PCR, but none of them have shown attempts to actively cool the matrix back down when necessary for other steps in the thermocycling. Some have implemented electric fans, or simply rely on a large volume of fluid surrounding the PCR sample container to act as a heat sink to cool the sample back down. In fact, optical refrigeration of enzymes has not been demonstrated yet at all in any context, much less in PCR applications. I believe we could use YLF to effectively address both the heating and

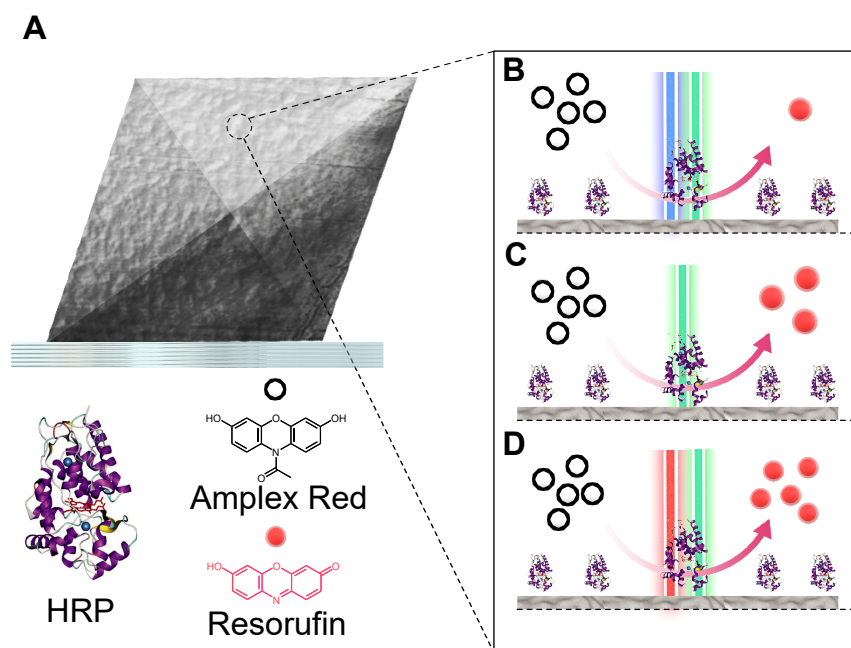


Figure 2.2: Modified turnover rate of HRP bound to YLF during optical heating and cooling. (A) Magnified representation of a typical HRP YLF crystal, showing the surface roughness caused by the silica coating, an X-ray crystallography model of the target enzyme HRP, and the chemical structures of the substrate and its transformed state. The following panels show a magnified representation of the system at the surface of the HRP YLF during a typical rate experiment when being optically cooled with a 1020 nm laser (B), when only illuminated by the 532 nm signal laser (C), and when being optically heated with a 980 nm laser (D).

cooling needs of PCR using only one material and two different laser wavelengths. A figure demonstrating this concept is shown in Figure 2.2.

This work presents an optical method of locally heating and cooling horseradish peroxidase enzymes that are covalently bound to the surface of modified YLF crystals. A synthesis is proposed and performed in order to functionalize the surface of the YLF crystals in such a way that HRP will readily bind to them, and the cooling ability of the crystals is shown to be maintained in spite of the coating and enzymes upon their surfaces. Work is also per-

formed to demonstrate the effect of temperature on HRP activity to ensure we should see a difference in activity based on a change in temperature of the HRP-bound YLF (HRPYLF) solution and can also use those results to estimate an actual temperature difference attained by the particles during rate experiments. Enzymatic activity rate experiments are also performed in order to determine if the binding of the enzymes has disrupted their function in a significant manner, and then rate experiments are performed in the presence of different laser wavelengths and dosages to examine the effect of the crystals' cooling and heating on the enzymes' functionality. The heat transfer occurring in the system is also briefly discussed.

2.2 Materials and Methods

2.2.1 Synthesis of Ytterbium-Doped Yttrium Lithium Fluoride

1-5 μm Size Crystals

This procedure closely follows that of Roder et al.[10] The following synthesis describes the creation of 10%-ytterbium doped yttrium lithium fluoride through a hydrothermal method. Yttrium nitrate ($\text{Y}(\text{NO}_3)_3$) and ytterbium nitrate ($\text{Yb}(\text{NO}_3)_3$) were both of 99.99% purity and were dissolved in Millipore water to form aqueous solutions of each compound. 7.2 mL of 0.5M $\text{Y}(\text{NO}_3)_3$ and 0.8 mL of 0.5M $\text{Yb}(\text{NO}_3)_3$ were mixed with 1.17 g of EDTA and 314 mg of lithium hydroxide to form Solution A. Solution A was stirred at 80 °C for 30 minutes or until EDTA was fully dissolved. A second solution, Solution B, was formed by dissolving 0.21 g of lithium fluoride and 0.68 g of ammonium bifluoride were dissolved in 7 mL Millipore water. This solution was also stirred at 80 °C until the lithium fluoride was completely dispersed. After the required length of time for stirring, Solution B was added dropwise to Solution A and the resulting mixture was allowed to stir for an additional 30 minutes at 80 °C. The mixture was then placed in a Teflon-lined autoclave and heated in

an oven to 220 °C for 72hr. After its time in the oven, the resulting solid is washed and centrifuged with ethanol 1x, deionized water 2x, and ethanol 1x, then allowed to dry. The white powder is then calcined at 300 °C for 2 hr in a tube furnace and the final product is obtained.

5-20 μm Size Crystals

To obtain YLF crystals of a significantly larger size than those produced by the previous procedure, a similar method was used with some alterations. $\text{Y}(\text{NO}_3)_3$ and ytterbium chloride (YbCl_3) were dissolved in Millipore water to form 5M aqueous solutions of each. 7.2 mL of the yttrium solution and 0.8 mL of the ytterbium solution were mixed together to form Solution A and guarantee a 10% Yb dopant concentration, as above. This solution was stirred at 80 °C for 30 minutes. Solution B was formed by dissolving 2.1 g of lithium fluoride and 8.8 g of ammonium fluoride in 7 mL of water. Ammonium fluoride was used in place of ammonium bifluoride due to the increased mass of reactants required for this synthesis, and using such high quantities of ammonium bifluoride greatly increases risk of hydrofluoric acid formation during synthesis. The equivalents of fluorine have been adjusted accordingly. Solution B was also stirred at 80 °C for thirty minutes to ensure significant dispersal of the lithium fluoride. Solution B was then added dropwise to Solution A, then allowed to stir for 30 additional minutes at 80 °C. The resulting solution volume was much larger than the previous procedure due to the 10x increase in reagent mass and/or concentration, and so the solution was divided and poured into two Teflon liners to avoid leaking. Both liners were sealed within steel autoclaves and placed in an oven at 220 °C for 72 hours. The resulting solids were recombined, washed and centrifuged with ethanol 1x, deionized water 2x, and ethanol 1x before being allowed to dry. The white powder was then calcined at 300 °C for 2 hr in a tube furnace. The final yield was close to 15 grams, which is roughly 10x the yield of

the previous procedure and matches the 10x increase in reagent mass and/or concentration. For the purposes of future experiments, either size of previously synthesized YLF crystals can be used and so will be referred to generically as “YLF.” If a specification is necessary, it will be written out.

Silica Coating and Characterization

This procedure is adapted from the process found in Patil et al for buildup of mesoporous silica nanospheres onto nanotubes.[72] For the first step, 1 g of the previously synthesized YLF is finely dispersed in 50 mL of ethanol through sonication. The resulting solution is stirred at 400 rpm as 1 mL of deionized water, 2 mL of tetraethyl orthosilicate (TEOS), and 4 mL of 14% NH_4OH are added to the mixture. This solution is allowed to stir for 30min at room temperature before the coated solid is washed and centrifuged with ethanol 1x, deionized water 2x, and ethanol 1x, resulting in a thin layer of mesoporous silica microspheres that deposited on the surface of the YLF. The cleaned solid is then allowed to dry. The presence of silica nanospheres on the surface of the crystals was confirmed by scanning electron microscopy (SEM) performed on a ThermoFisher Scientific Apreo SEM in the Molecular Analysis Facility at UW.

Amine and Aldehyde Functionalization and Characterization

This procedure is a modified version of the method found in Chen et al.[73] The cleaned solid from the previous step is redispersed in ethanol via sonication and stirred at 400 rpm. 2 mL of (3-aminopropyl) trimethoxysilane (APTMS) are added to the solution and the mixture is allowed to stir for 24 hr at room temperature, during which time the silica microspheres on the YLF surface become functionalized with amine groups. After the overnight stir, the solid is washed and centrifuged as described previously for the silica coated YLF. The solid is then

allowed to dry for purposes of characterization and accurate weighing in future steps. 30 mg of the new amine-YLF is then dispersed into 15 mL of a pH 7.4 phosphate buffered saline solution (PBS). The mixture is stirred at 300 rpm, then 15 mL of a 2.5% glutaraldehyde solution is added and the mixture is allowed to stir for 2hr at room temperature. The resulting solid is washed and centrifuged as described previously and then allowed to dry. This leaves the former amine-functionalized silica spheres now aldehyde-functionalized.

Both the amine-functionalized and the aldehyde-functionalized YLF crystals, once dried, were characterized using Fourier-Transform Infrared Spectroscopy (FTIR) performed with a Bruker VERTEX 70 instrument housed in the Materials Science and Engineering department's undergraduate laboratories. The attenuated total reflection (ATR-FTIR) attachment was used to be able to observe the solids by themselves, without the influence of any solvent.

Enzyme Coating and Characterization

The enzyme introduction step occurs in conjunction with the aldehyde functionalization step, provided that the aldehyde-functionalized product is not needed for any FTIR characterization. After the 2hr stir step mentioned in the previous section, an amount of lyophilized HRP is added to the solution in accordance with a desired concentration in terms of activity units per mL. The activity unit is defined as the amount of enzyme needed to convert one micromole of substrate in one minute. This mixture is moved to a refrigerator in order to stir at 300rpm at 4 °C for 96hrs. The mixture is then washed and centrifuged with fresh PBS at 5000rpm for 2 minutes, 3x to remove any remaining free-floating HRP in solution that may obfuscate the activity of enzyme directly bound to the surface, resulting in enzyme-coated crystals I will call HRPYLF.

2.2.2 Optical Refrigeration Measurements

All optical refrigeration measurements were performed on a lab-built inverted microscope setup and spectroscopy setup. Spectroscopy data was collected using a Princeton Instruments Acton SpectraPro 500i spectrometer and its corresponding software, Lightfield.

Mean Fluorescence

Mean fluorescence measurements are performed using a sample chamber made up of a glass microscope slide and slide cover, held together by a double-sided adhesive spacer with a thickness of $100\ \mu\text{ m}$. A small aliquot of the sample of interest is dispensed into a well inside of the spacer, holding the sample in place. Prior to any measurements, careful background correction is performed using a sample chamber containing only the solvent that the crystals will be dispersed in to eliminate any potential fluorescence of the glass under 1020 nm irradiation. The crystal of interest is placed in the path of the 1020 nm diode beam, and then the mirror path is adjusted to allow the fluorescence signal of the Yb^{3+} ions to the spectrometer via a fiber coupling. The resulting spectrum is then run through a Python script written by a previous member of the research group, which integrates the area underneath the curve of the spectrum to find the expectation value, or mean wavelength value of the spectrum. Multiple measurements are taken using constant laser power that is modulated by a neutral density filter wheel containing filters of five different thicknesses, so that mean wavelength value can be plotted against laser power to observe whether the mean wavelength value redshifts or blueshifts with increasing laser power.

Calibration of Mean Fluorescence Measurements

The data obtained using the method and code described above will not output a temperature shift value like the cold Brownian motion code is capable of. In order to put our mean wavelength shift in terms of temperature, a separate calibration measurement must be performed on the sample of interest. An aliquot of the sample of interest dispersed in water is dispensed into the chamber of a temperature-controlled perfusion chamber. The perfusion chamber is then placed on the stage of the inverted microscopy setup similarly to previous samples and hooked up to the temperature controller. Two thermocouples are placed: one inserted into the metal ring of the chamber and one taped down onto the glass surface of the sample area as close as possible to where the mean fluorescence measurement is taking place. This allows us both control over the chamber's temperature while also granting insight about the actual temperature near the sample itself. Mean fluorescence measurements are then taken at a constant laser power (7.6mW) and at a known temperature, starting at 25 °C and increasing in increments of 2.5 degrees up to a maximum of 45 °C. Mean fluorescence can then be plotted against temperature, generating a dataset that can be fit with a linear trendline. This trendline can be used to extrapolate temperature change from mean wavelength shift, allowing for more practical analysis of the cooling power of the crystals.

2.2.3 Enzymatic Activity Measurements

Temperature Dependence of HRP

In order to confirm the presence of a notable change in enzyme activity based on changes in temperature, temperature-controlled UV-vis measurements were performed using an Agilent 8453 Diode Array UV-Vis Spectrophotometer housed in the University of Washington Chemistry Department. This spectrophotometer has a Peltier heating and cooling system within

its cuvette holder that can hold a sample at a desired temperature during the measurement. To perform a measurement, first the cuvette holder's temperature was set to the desired value. Then, 1.5mL of a solution of 100 μ M Amplex Red and 1 mM hydrogen peroxide was added to the quartz cuvette to reach the desired temperature. Once the temperature was reached, 1.5 mL of an HRP solution with 50 mU/mL of activity was added to the cuvette and the solution was rapidly mixed via uptake and dispensing from a pipette. Timed measurements then began, with a measurement taking place every 20 seconds looking at the absorbance of the mixture at $\lambda = 571$ nm. Trials were performed at 10, 15, 20, 25, and 30 $^{\circ}$ C to examine a range of feasible temperatures the enzymes could reach when bound to the YLF crystals.

Activity Measurements of Bound HRP

In order to compare the activity of the HRPYLF bound enzymes versus that of their free-floating counterparts, additional UV-vis spectroscopy measurements were performed using a PerkinElmer Lambda 950 spectrometer housed in the Research Training Testbed at the University of Washington.

To perform a measurement, 1.5 mL of a solution of 100 μ M Amplex Red and hydrogen peroxide of various concentrations (25, 75, 150, 250, 500 μ M) was dispensed into a quartz cuvette. Then, 1.5 mL of a solution of either free HRP or HRPYLF crystals with an expected enzymatic activity of 100 mU/mL is added to the cuvette. The solution in the cuvette is then rapidly mixed by inversion and placed inside the cuvette holder of the spectrometer as quickly as possible, usually taking around 5 seconds. The spectrometer then takes measurements of the absorbance of the solution at $\lambda = 570$ nm every 30 seconds, generating an enzymatic activity rate dataset. One measurement of this type was performed for each unique concentration of hydrogen peroxide for the purpose of creating a Michaelis-Menten

plot that would provide information about key enzyme activity metrics to determine the effectiveness of the bound enzymes compared to the free ones.

Bulk Optical Cooling Activity Rate Measurements

The sample chamber for bulk HRPYLF measurements is prepared according to the procedure outlined in the cooling measurements sections previously; the only difference is the preparation of the sample solution. The sample solution is made up of 25 μL of a 200 μM Amplex Red solution, 25 μL of a 2 mM hydrogen peroxide solution, and 50 μL of a solution containing HRPYLF crystals. The two solutions not containing crystals would be pipetted into the bottom of a microcentrifuge tube first, then the HRPYLF solution would be dispensed carefully onto the side of the tube, which is then laid horizontally on a benchtop to prevent mixing of the two solutions until ready. A 532 nm diode laser is focused upon where the sample will be placed for control (non-cooling) measurements. For cooling measurements, a 1020 nm diode laser that is coaligned with the 532 nm laser would also be on and focused upon where the sample will be placed. The powers of each laser will be mentioned with their corresponding datasets in the Results and Discussion section. A background measurement is taken using a sample chamber filled with only phosphate buffered saline to remove any effects from the glass chamber from the obtained spectra. Once the lasers and data collection software are prepared, the microcentrifuge tube containing the sample solution is closed and rapidly shaken in order to mix the separate solutions together. At the same time, a stopwatch timer is started to track the progress of the reaction. The mixed sample solution is then pipetted into the sample chamber, which is then sealed and placed into the path(s) of the laser(s). This process takes place within the first 60 seconds of the solutions being mixed so that the first fluorescence spectrum from the converted resorufin can be captured by the spectrometer. Fluorescence measurements are taken at 60, 90, 120, 150, 180, 210,

240, 270, 300, 420, 600, and 900 seconds to complete one trial. The fluorescence intensity at $\lambda = 585.3$ nm is taken from the data and plotted against time to generate an enzyme activity rate plot similar to those of the previously described UV-vis measurements.

Other measurements were performed following this method using uncoated YLF crystals and YLF crystals that were not doped with ytterbium in place of HRPYLF in order to rule out the possibility of autocatalysis of the Amplex Red reagent caused by the lasers or any features of the crystals themselves.

Single Crystal Optical Heating and Cooling Activity Rate Measurements

To be able to examine a single HRPYLF crystal repeatedly rather than have to make a new sample for each measurement, a system that could flow fresh reagents to the HRPYLF crystals and then wash the reacted reagents away was developed. Glass-bottom channel slides with a channel height of 200 μm and Luer adapters were purchased from Ibidi for use with latex tubing and a syringe pump for these experiments.

For a set of single crystal experiments, HRPYLF solution is pipetted into the channel until the solution completely fills the channel slide. The slide is then placed on the stage in the path of the lasers of interest (532, 980, and 1020 nm) and hooked up to the tubing that connects the channel slide to the syringe pump and the solution reservoir. Fresh phosphate buffered saline is then pulled into the chamber by the syringe pump using a flow rate of 7 mL/minute. This step is performed to flush any free-floating HRPYLF crystals out of the chamber to ensure that only crystals stuck to the glass at the bottom of the chamber remain, so that we know we will not lose the crystal of interest due to the flow of reaction mixture into the chamber throughout multiple trials. The next step is to find an HRPYLF crystal that cools when irradiated by a 1020 nm source. Mean fluorescence measurements are performed on single crystals found in the chamber until a crystal with a cooling trend is found.

Background measurements are also performed at this time to remove effects from the glass and any erbium contaminants present from the ytterbium precursor caused by difficulties with purification of lanthanides, which can appear in the fluorescence measurements when a 980 nm source is used.[74] After background correction is finished, the tube is moved from the phosphate buffer to the reaction mixture: a solution of 250 μ M Amplex Red and 1 mM hydrogen peroxide. This solution is also pulled toward the reaction chamber at a rate of 7 mL/minute until it is just about to enter the channel. The flow rate is then changed to 500 μ L/minute to conserve both the crystal of interest and reaction solution. Once reaction solution has entered the channel, fluorescence measurements can begin.

There are three reaction conditions of interest we are testing: control (no purposeful heating or cooling, 532 nm irradiation only), heating (532 and 980 nm irradiation), and cooling (532 and 1020 nm irradiation). In some trials, a shutter was placed in the path of the 532 nm laser in order to cut the overall dose of high energy radiation to the enzymes. 980 and 1020 nm irradiation is always constant. The desired lasers for each reaction condition are turned on, and fresh reaction solution is pulled into the chamber until a stable baseline value of resorufin fluorescence is attained. The flow of solution is then stopped and automatic timed fluorescence measurements are started. Measurements are taken every 60 seconds for a total of 10 minutes with the shutter closed for 53 seconds and open for 7 seconds to allow for saturation of the present resorufin molecules and for the spectrum to be collected. The data is then worked up in the same way as described in the previous section to generate enzyme activity rate plots for each trial.

2.3 Results and Discussion

2.3.1 Synthesis and Surface Functionalization of Yttrium Lithium Fluoride

The success of each completed step was confirmed by a couple of different methods, depending on the step in question. For the silica coating step, scanning electron microscopy (SEM) is useful to observe the microspheres themselves that have built up upon the YLF crystals. SEM images of the silica coated small YLF are shown in Figure 2.3a-c, while images of similarly coated large YLF are in panels d-f. Initial experiments performing the silica buildup involved some trial and error to discover the ideal amount of time for coating, as it was unknown how readily the silica would attach to the YLF. As it turns out, it attaches very readily, and a stir time of only thirty minutes was required when combined with centrifugation. One interesting attribute of the silica coating post-centrifugation is that it prefers to build up on the truncated points of the bipyramidal structure, which we believe to be the [001] plane of the crystal structure. A possible reason for the preferred buildup on this plane is that the fluorine atoms are more readily accessible at this surface. The fluorine atoms can be substituted by hydroxyl groups in the presence of water (which is present both in the reaction mixture and in air), which the tetraethyl orthosilicate will preferentially bind to the hydroxyl groups over the elements present at other crystal faces. One other attribute of note is how thin the silica layer is on the surface of the YLF crystals. Silica will heat under the laser irradiation necessary for laser cooling, which could cause the silica's heating to overwhelm the YLF's cooling ability. Additionally, the silica coat could end up catching photons that are exiting the Yb^{3+} atoms in the crystal structure, absorbing them in a nonradiative manner and converting them to additional heat. As such, to maintain the cooling capabilities of the crystals, we want the silica coat to be as thin as possible. It is encouraging to see that, when following the optimized procedure, the coated surfaces only have a single layer of spheres

built up on them, indicating that this is the thinnest possible silica coat we can achieve. The spheres also appear to be quite small, approximately 25 nm in radius, which is significantly smaller than the size of sphere they achieve in the literature source this procedure was adapted from.⁴⁰ This could be due to the fact that they allow their silica formation and buildup step to stir for longer, allowing more growth of the spheres that this work cannot afford due to the optical cooling concerns mentioned above. It is possible that this step could be optimized further to use smaller spheres or thinner coats produced by a different method, which is a possible direction of future work.

With confidence in the success of the silica reaction, the amine and glutaraldehyde steps were performed, but these small chemical changes would not be indentifiable via SEM. Therefore, FTIR is currently the method used to determine the result of these functionalization experiments, and a resulting spectrum is shown in Figure 2.3g. Evidence of all three functionalization steps can be seen in these two spectra. The silica is seen in the large peak at 1070 cm^{-1} and in the broad stretch centered at 3400 cm^{-1} , which also contains the signal for any unreacted amine groups. Lastly, there is a small bump present at 1600 cm^{-1} that indicates the presence of the carbonyl group within an aldehyde, provided by the glutaraldehyde. This aldehyde feature is absent from the spectrum of silica-coated YLF and so we can be certain that the peak comes from the presence of the glutaraldehyde and isn't an artifact of some kind from the silica-coated crystals.

The final step of the surface modification of the YLF crystals is to introduce our HRP enzymes, which was done using enzymatic activity assays with our substrate of interest Amplex Red. After cleaning and centrifugation, when the crystals were introduced to a solution of Amplex Red and hydrogen peroxide, the solution rapidly changed from clear to pink, confirming both that enzymes had remained in solution, bound to the crystals, and

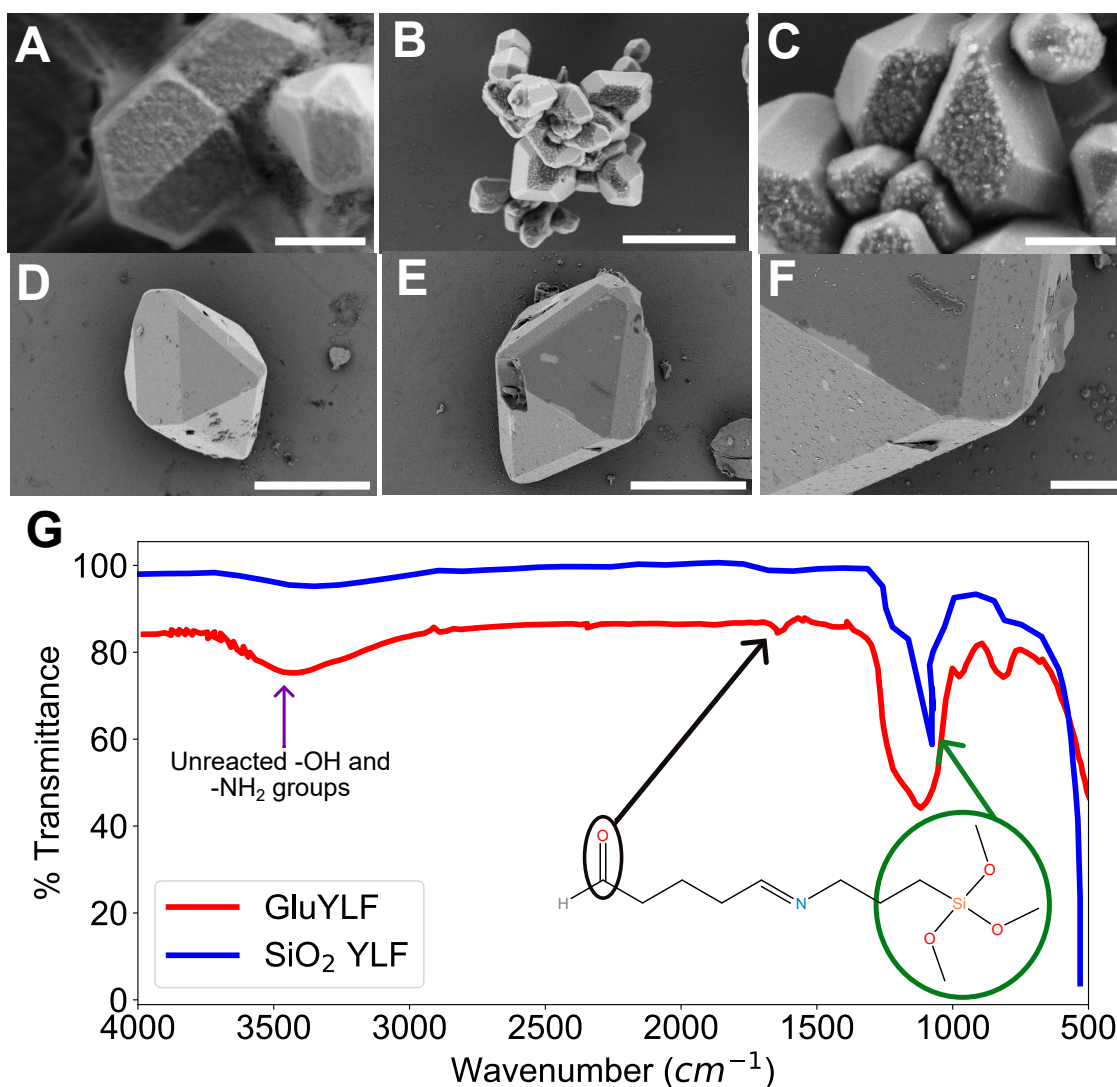


Figure 2.3: Characterization of the HRPYLF synthesis. (A) SEM image showing a YLF crystal showing a thick coating of silica that would render optical cooling nearly impossible. Scale bar is 500 nm. (B-C) SEM images of YLF crystals that underwent proper workup to remove excess surface silica, minimizing heating due to silica coating. Scale bars are 2 μm and 500 nm respectively. Images courtesy of Alex Bard. (D) SEM image showing a YLF crystal prior to any surface coating, showing the typical bipyramidal shape. Scale bar is 20 μm . (E-F) SEM images of a YLF crystal that has been surface functionalized up to the amine step. The surface texture has changed, appearing rougher than the uncoated crystal. Scale bars are 20 μm and 5 μm respectively. Images courtesy of Lars Forberger (G) FTIR spectra showing the appearance of glutaraldehyde after that synthesis step, along with other functional groups related to the presence of silica.

that they were still functional. Testing that determines how their activity compares to their unbound brethren will be discussed in a later section.

The next step of characterization is to determine that the HRPYLF crystals can still cool despite the coating we have now wrapped our cooling crystals in. A table and histogram showing cooling data from 15 cooling crystals measured out of 25 different total crystals are shown in Figure A.1. As can be seen, the HRPYLF crystals are still capable of local cooling, sometimes by as much as 25 °C, though this is likely an outlier. On average, the crystals cool between 5 and 10 °C, which is still a significant change. Some crystals do demonstrate heating tendencies, but methods for isolating cooling crystals out of a mixed sample will be discussed in later sections. However, these temperature changes will not mean anything if it is not a large enough shift to induce a change in the activity rate of our test enzymes, and so the dependence of HRP activity on temperature must next be probed.

2.3.2 Rate of Reaction of the Enzyme with Temperature

The obtained temperature-dependent UV-vis data is shown in Figure 2.4. The measurement intended to be taken at 20 seconds in the 10 °C trial is missing due to an error when first developing the measurement technique, but the rest of that temperature's dataset supports the trend I wish to discuss well enough that I believe it is worthwhile to include. As can be clearly seen, as the temperature of the solution increases, so does the rate of reaction of the enzymes. The sample held at 30 °C shows the highest rate of reaction, which aligns well with values from the literature for optimal activity temperature for HRP.[75, 76] It can also be seen that even temperatures as low as 10 °C do not completely halt enzymatic activity, and so even if the enzymes are attached to a crystal with very high cooling efficiency, we should still see enzymatic activity taking place through our fluorescence measurements. This dataset also demonstrates that there is a clear difference in enzymatic activity between temperatures

with only 5 degrees between them, and so even when attached to a less efficient YLF crystal a difference in activity rate should be visible. This dataset provides a strong foundation for this project by proving not only that HRP activity is strongly dependent on temperature, but also that we can observe a significant difference in activity with only small changes in temperature that are feasible to achieve with our doped YLF crystals in an aqueous medium.

An interesting feature of the data is the decrease in absorbance shown at the later time points in all trials except for the 10 °C trial. The change is also possible to see visually in both cuvette and in the sample chambers used for optical tests, as the solution changes from colorless and transparent, to translucent and very bright pink, to transparent and a much paler pink. An exact explanation for this phenomenon is currently unknown, but there are a few possible explanations. The first hint to an explanation

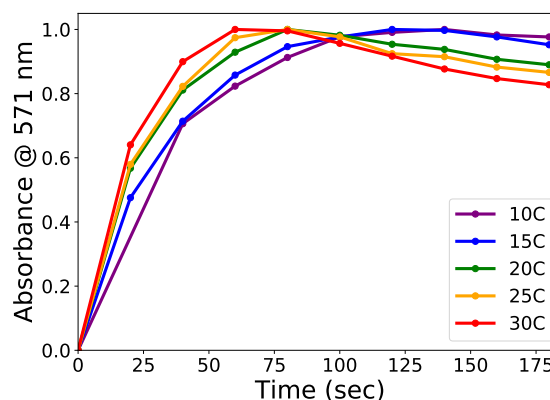


Figure 2.4: Temperature controlled UV-vis measurements of free HRP at a range of temperatures possible for our crystals to reach via laser irradiation.

is that this phenomenon is only seen when performing absorbance measurements, and not seen when performing fluorescence measurements. Although it is possible to measure the presence of resorufin using both spectrophotometric measurements and fluorescent measurements, fluorescence is a more useful method for detection.[77] It allows for lower detection limits, and if there is one major difference between the two techniques, it's possible that this could explain the decrease we observe at longer time points. Recent literature has observed that pi-pi stacking, which could be possible in resorufin due to the aromatic rings it contains,

can shift the absorption and emission bands of a molecule, though this would also in theory affect the fluorescence measurements in addition to the spectrofluorimetric effect we see.[78] Another possibility is the presence of a self-quenching effect that primarily affects the absorption at 570 nm but still allows the excitation that leads to fluorescence to take place at other wavelengths. Self-quenching effects that take place at high concentrations are well documented in fluorophores used in protein aggregation testing and other biological sensing applications.[79] Of these possibilities, the self-quenching effect is the hypothesis that is most likely to be true due to the wealth of literature discussing self-quenching effects in this type of fluorophore. Regardless, this phenomenon does not have any major impact on the optical cooling trials that will be discussed later in this manuscript.

2.3.3 Enzymatic Activity at the Yttrium Lithium Fluoride Surface

Now that we have qualitatively determined the enzymatic activity of our HRPYLF crystals, the next step is to quantitatively determine it. Data comparing UV-vis activity measurements of unbound versus YLF-bound HRP are shown in Figure 2.5a. As can be seen, our HRPYLF crystal sample shows reduced activity compared to the unbound sample, although in theory both samples should contain equal amounts of active enzyme. This is a highly concerning result, showing that there could be activity inhibition due to enzyme denaturation or steric blocking of the active site. However, it was also possible that the decrease in observed activity could be due to issues with the binding of the enzymes to the crystals. If not every single enzyme introduced to the crystals attached to them, then even though we added equal amounts to the free and bound enzyme solutions, the bound solution would demonstrate lower activity after the remaining free enzymes were removed via washing. Therefore, UV-vis data for creation of a Michaelis-Menten plot was collected, as that would tell us about important enzymatic parameters that can indicate the health and accessibility of our

enzymes independent of their concentration in solution. A Michaelis-Menten plot is formed by calculating the initial rate of reaction of the enzyme in units of converted substrate concentration over time, which for this work can be calculated from the slope of the enzyme rate plots multiplied by the molar absorptivity of resorufin. With measurements performed using varying substrate concentrations, you can generate a plot of enzymatic rate of reaction against substrate concentrations, which is the Michaelis-Menten plot. This data can be fitted to the following equation:

$$v = \frac{V_{max} * [S]}{K_m + [S]} \quad (2.1)$$

in which v is the reaction velocity, $[S]$ is the concentration of substrate, V_{max} is the maximum reaction velocity, and K_m is the substrate concentration at which the reaction velocity is one half of V_{max} . K_m in particular is a very useful value to know for your enzyme, as it describes how strong the affinity your enzyme and your substrate have for each other. The lower your value of K_m , the higher the affinity between enzyme and substrate. The Michaelis-Menten plot obtained for our HRPYLF crystals is shown in Figure 2.6b. A least squares regression fit is shown on this plot, from which we extract our K_m value of approximately 27 μM . The plot shows excellent agreement with the calculated data points, but this value is significantly lower than values found from the literature, which can range from 200 μM up to 4.0 mM.[80, 81] This technically implies that our bound enzymes have higher affinity for hydrogen peroxide compared to the free enzyme values listed in these references, though it is unlikely that the binding of the enzymes has increased their substrate affinity. It is possible that a true V_{max} was not reached, but the collected data shows agreement with the Michaelis-Menten model and the K_m value obtained in the realm of the lowest literature values, these samples were used for future laser-influenced rate experiments.

If the enzymes are still fully functional, this would imply that we did not achieve 100%

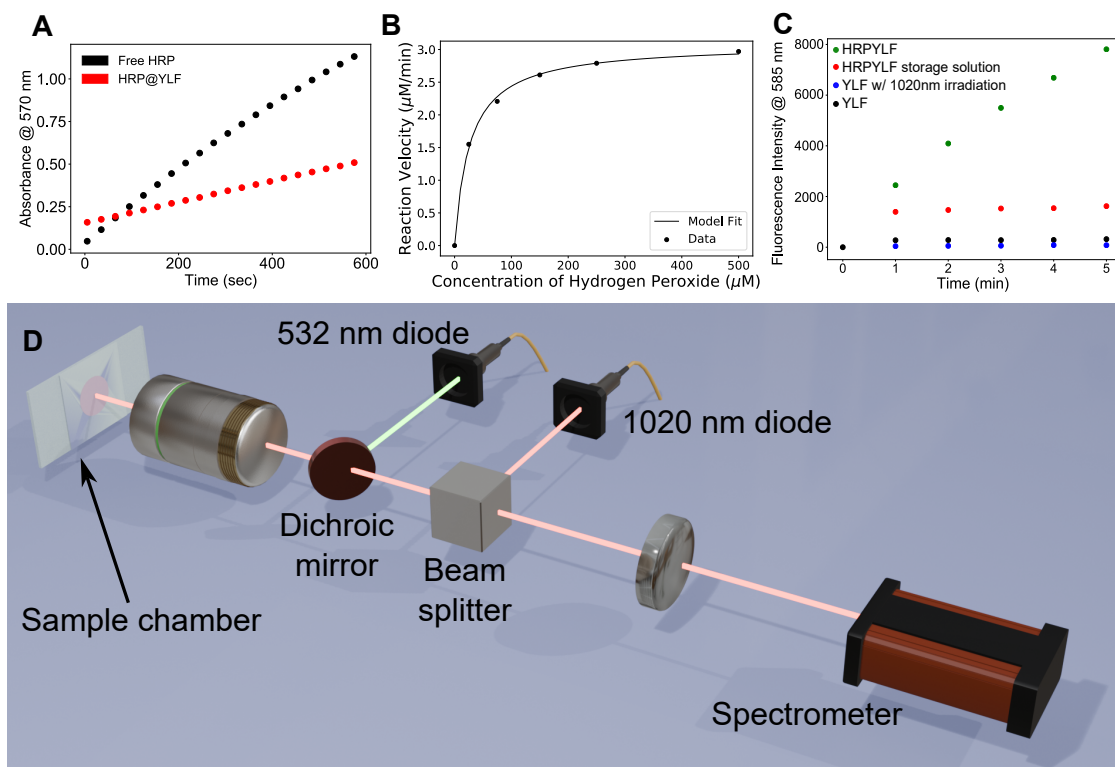


Figure 2.5: Enzyme function and control tests. (A) UVvis experiments comparing identical concentrations YLF-bound HRP with free enzyme. (B) Michaelis-Menten plot showing that enzymatic activity is not effectively reduced in the bound HRP. (C) A variety of fluorescence experiments performed to confirm that any observed HRP activity could only be caused by active enzyme attached to YLF crystals and not due to free HRP in solution nor any interactions between the laser and the crystals or substrate. (D) Simplified diagram of the optical setup used to obtain the results shown in C.

binding efficiency of the enzymes we introduced to our aldehyde-functionalized YLF. Based on the plot in 2.5a, we can see that the rate of reaction was reduced by approximately 67.7%, meaning that only one third of the enzymes bound to the crystals. The enzymes purchased from ThermoFisher have a unit/mg value of 248 U/mg, which means that in our 50 mL of 100 mU/mL (prepared via serial dilution), there are roughly 0.0202 mg of HRP present. When combined with the molar mass of HRP and Avogadro's number, this yields a value of 2.76×10^{14} individual HRP enzymes, meaning that 9.20×10^{13} enzymes bound to the crystals. The number density of YLF crystals is calculated using the value of 0.03 g present per 1.5 mL aliquot and the density of 10%-doped YLF (3.95 g/cm^3) to yield a total volume of YLF of 0.00759 cm^3 . Assuming the YLF crystals to be a cuboid with dimensions of $2.5 \times 2.5 \times 3.33 \text{ }\mu\text{m}$ based on CCD camera imaging, as has been done in previous work,[82] it is determined that there are roughly 3.64×10^8 YLF crystals present in the aliquot. This means there are, on average, 2.53×10^5 HRP enzymes on each YLF crystal. HRP enzymes are known to have dimensions of roughly $4.3 \times 4.8 \times 5.8 \text{ nm}$ from x-ray crystallography data,[83] and so the theoretical maximum number of enzymes that could fit on one YLF crystal is 2.22×10^6 . This implies only an approximate 10% surface coverage of our YLF crystals compared to what is theoretically possible, ignoring possible steric effects that would spread out the enzymes or the very slight increase in the amount of space needed per enzyme due to hydrodynamic diameter. One possible reason is that, as can be seen in the SEM images in Figure 2.3, the silica nanospheres do not appear to collect on all surfaces of the YLF, selectively growing along the truncated points of the pyramid. With only partial silica coverage, we can only achieve partial enzymatic coverage as well. It is also fully possible that the stir time allowed for this reaction needs to be increased in order for more collisions to occur and the amine-aldehyde reaction to be successful. However, we are already pushing up against reasonable

synthesis time constraints by allowing stirring for upwards of 96 hours, so increasing the concentrations of the reagents of interest would be best for future work. Even with low surface coverage, enzymatic activity is still detectable with our methods.

2.3.4 Enzymatic Rate Experiments Using Optical Refrigeration and Heating

With confirmation that the enzymes are still working well while bound to our YLF crystals, the next step is to begin attempts to influence their behavior via optical methods. An important step to take in order to ensure the quality of our data was to perform a number of control measurements to rule out the possibility of autocatalysis of Amplex Red to resorufin due to any photolysis of hydrogen peroxide caused by the 1020 nm laser, which is noted as possible in the literature for photons generated by the Yb^{3+} ions in the YLF crystals.[84] Another important condition to test was to test the supernatant of the HRPYLF storage solution, both to ensure that no free HRP remained that could cause resorufin formation as well as test for any degradation of the silica coating over long periods of storage time. Tests were performed using sample chambers containing just reaction solution, chambers containing reaction solution and uncoated YLF, and chambers containing reaction solution and the HRPYLF sample supernatant with 532 nm and 1020 nm irradiation. A summary of these results can be found in Figure 2.5c, alongside a simplified optical diagram showing the setup used to obtain these measurements. As can be seen, for all of these control trials, we see little to no enzymatic activity in the control samples compared to samples containing HRPYLF crystals. This is strong confirmation that any activity we see in the fluorescence trials to come can be attributed to HRP enzymes that are bound to the surface of our crystals.

The next step is to attempt optical cooling of the crystals and see what change is induced in the enzyme activity following the method outlined in a previous section. A great deal of

trials were performed during this phase of the project, many of which were inconclusive and showed no major difference between the two experimental conditions, those being 532 nm irradiation only (control) and 532 and 1020 nm irradiation (cooling). The typical laser powers for each laser is 200 μ W for the 532 nm laser and 30 mW for the 1020 laser. Some measurements obtained during this period appeared promising and shows the trend we hope to see, but unfortunately, the mean fluorescence data captured post-activity rate measurement did not align with the trend seen in the activity measurement. Crystals that were being tested under the "cooling" conditions of 532 nm and 1020 nm irradiation demonstrated blueshifts indicative of heating rather than the cooling that was believed to be seen. This in particular made clear that the current experimental setup would need to be changed in order to avoid wasting more data, reagents, and time.

A key issue that became apparent during this phase of the project is the amount of difficulties caused by sample chamber used for these trials. A new chamber has to be made for each individual trial, meaning that the crystal being examined also changed with every single trial. Based on the mean fluorescence data we obtained, this could mean that the crystal of interest is cooling by 5 °C, 25 °C, or may not even be cooling at all if we were unlucky. Because the HRPYLF crystals have to be mixed with the Amplex Red and hydrogen peroxide solution prior to the sealing of the chamber, we also cannot perform mean fluorescence measurements prior to the activity rate measurements to confirm that the selected crystal cools under 1020 nm irradiation. Another issue posed by these sample chambers is that, because of the chamber preparation method, there are a large number of crystals present within them, and are only able to cool one crystal at a time with the laser. This means that only a small proportion of the enzymes contained in the chamber are affected by the cooling laser, and the activity of the majority of enzymes would be unaffected. Their conversion of Amplex Red

to resorufin, and the resulting diffusion of resorufin into the path of our 532 nm laser, could obscure any small change in the enzymatic activity of the enzymes on the chosen crystal by sheer number. As such, a new method had to be developed in order to ensure that multiple trials could be performed on a single crystal, with a known cooling trend, and that only the activity of the chosen crystal is being recorded by the spectrometer.

A system involving microfluidic flow chambers was developed to address the concerns listed in the previous paragraph by allowing us to select a crystal that has adhered to the glass and will remain in place throughout multiple trials, to perform mean fluorescence measurements on the crystal of interest prior to introduction of reaction solution to confirm its cooling capability, and to remove crystals we are not interested in via the flow of fresh phosphate buffered saline. A simplified optical diagram of the flow setup and a zoomed in view of the sample chamber are shown in Figure 2.7a and b. Although the flow system addresses the challenges of the prior method effectively, it also comes with its own difficulties that could not be foreseen from the previous setup. Initial data from trials testing if heating would impact the rate of enzymatic reaction (the HRPYLF crystal receives 532 nm and 980 nm irradiation) appears highly promising, showing a very large increase in activity rate as seen in Figure A.2a. However, when these trials took place, I performed all three of the heating trials first and then performed all three of the control trials (532 nm irradiation only). Upon further examination of the obtained data, a harsh negative trend appeared in total resorufin fluorescence intensity with each successive trial. The intensity of the final data points from each trial are plotted in successive order in Figure A.2b, and the data shows a negative exponential trend. This was not a phenomenon we had witnessed during the bulk measurements due to each crystal and chamber only being used once, and so a fair deal of testing went into determining how much of the 532 nm laser dosage needed to

be cut to avoid photothermal damage to the enzymes over many successive trials. In the end, we determined that it wasn't possible to fully prevent the damage to the enzymes. We could greatly reduce it, however, through the use of a shutter in the path of the 532 nm laser and preserve data quality and comparability by alternating between experimental conditions rather than performing them in blocks so that each condition experiences roughly equal overall deterioration.

Another issue discovered during the course of these trials was the stability of the 532 nm green laser we had been using to collect signal from the resorufin. It had been noted across multiple trials using the new flow setup that the collected rate data was less precise than the data obtained with the previous setup. The signal often increased and decreased between data points seemingly at random, although the overall trend remained increasing. This was leading to enormous difficulties in obtaining data of statistical value, as the data at each time point being averaged could have a very wide spread. At first, it was believed that this was due to a leak in the flow system somewhere that was inducing some type of fluid flow in the chamber during experiments, carrying freshly produced resorufin away from our measuring point and adding very irritating convection considerations to any heat transfer analysis. After careful refurbishing of the flow setup (replacing tubing, rewrapping the tubing tightly with Parafilm at the interfaces with the Luer adapters, wrapping tape around the Luer inlet ports of the sample chambers, and even fully swapping sample chambers), introduction of fluid to the chamber, and leaving it overnight to see if it held, it was determined that the flow setup was not the problem and could hold liquid without leaks for far longer than was necessary for a day of trials. Attention next turned to the laser source to ensure that it maintains a stable power level throughout the experiments. When initially measuring and noting the input power during each day of experiments, it had always appeared stable enough for that

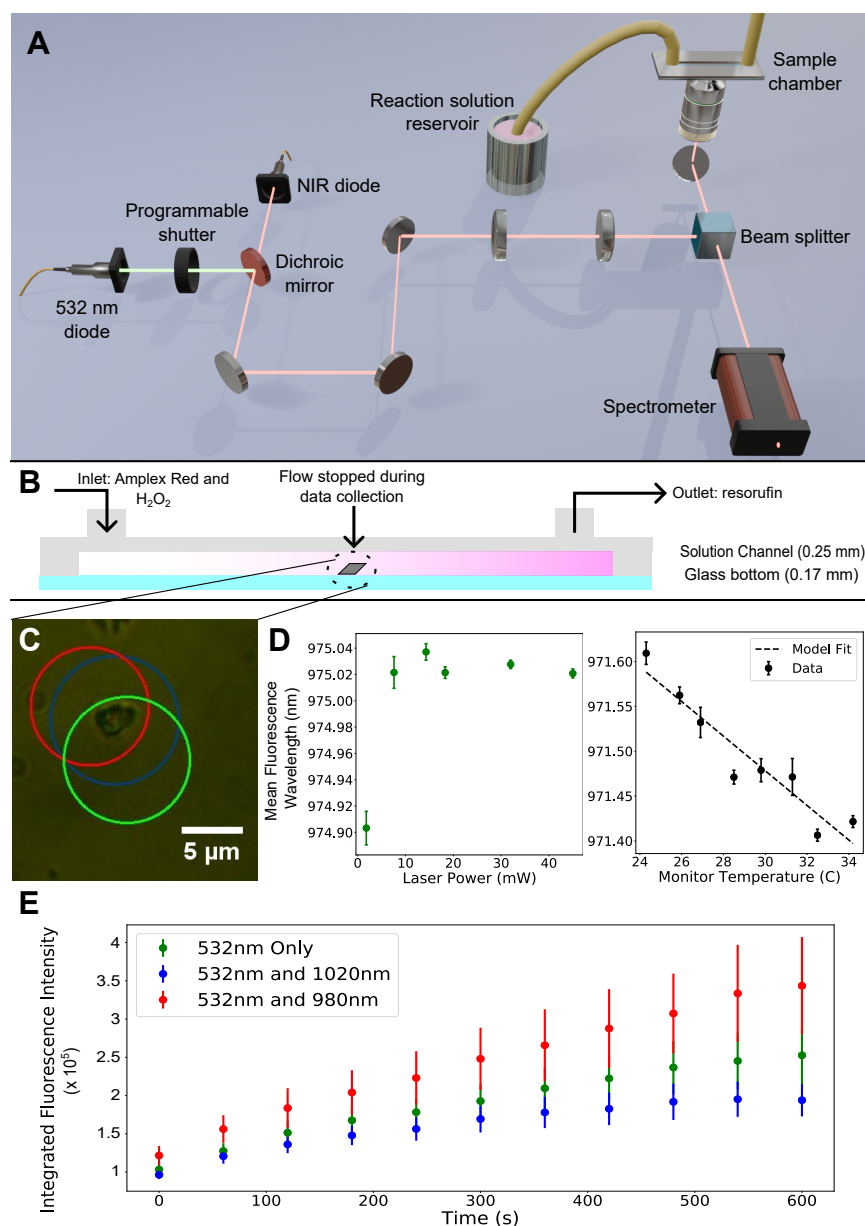


Figure 2.6: Summary of HRP YLF rate experiment setup and results. (A) Simplified diagram of the optical setup used during enzyme rate experiments. (B) Diagram of the flow chambers used for rate experiments, including relevant dimensions. The arrows indicate direction of flow when clearing the sample chamber of resorufin from previous testing and introducing fresh reaction mixture to the crystal of interest. (C) Camera image of the crystal the following results were obtained from. The colored rings indicate the locations of each laser diode spot, showing the close coalignment of the lasers (green = 532 nm, red = 980 nm, blue = 1020 nm). (D) Mean fluorescence data showing cooling of the crystal shown in (C) and a calibration curve from the same sample that allows for conversion from mean fluorescence shift to temperature change. Use of the calibration curve indicates this crystal cools by roughly 5 $^{\circ}C$. (E) Plotted data from rate experiments performed under control (green), heating (red), and cooling (blue) conditions. Data points are the average of four trials for each condition and error bars show the standard error across those trials.

quick measurement. When the power was measured over several minutes, it immediately became clear that this laser was not able to maintain a stable power level. Plots showing the instability of the green laser compared with the two NIR lasers are shown in Figure A.3. The optical table was then adjusted so that a 532 nm diode laser used for Raman experiments, with a well characterized single mode and stable power output, would be used instead of the previous diode.

With the new laser and an enzyme-conserving sample collection scheme in place, the data shown in Figure 2.7c-e was collected. The three different trial conditions of cooling (O), control (C), and heating (H) were performed in a pattern of O-C-H-H-C-O, repeated twice. This alternating pattern distributes the observed damage to the enzymes over time across the three trial conditions more equally, meaning that the conditions are more comparable than if they were to be performed in blocks. Using the improved capabilities of the flow setup compared to the previous bulk measurement setup, a cooling crystal was able to be selected before beginning trials. The crystal used for the presented rate result is shown in Figure 2.7c. This is an image from the CCD camera present along the path, and the colored rings represent the approximate locations of each laser (green = 532 nm, red = 980 nm, and blue = 1020 nm), showing that the NIR lasers are closely coaligned and the green laser is coaligned closely enough to pick up signal from the area surrounding the crystal of interest. Panel d shows the mean fluorescence cooling data collected from the particle and the calibration curve used to deduce the exact temperature shift caused by the YLF crystal, which is roughly 3 °C in magnitude. This may not seem like much, but it was enough to induce the change in rate of reaction shown in Figure 2.7e, where each point is the average of four trials and the error bars represent the standard error. As can be seen, the heating trials show an increased rate of reaction relative to the control, which show an increased

rate of reaction relative to the cooling trials. There is, admittedly, some overlap between the error bars that can be attributed to the drop in fluorescence intensity brought on by the degradation of the enzymes across a full day of experiments. This spread is unfortunately at this time unavoidable, but even in spite of the possible statistical analysis questions this may lead to, I still believe the result to be valid. This result has been replicated with two additional HRPYLF crystals showing similar laser cooling magnitudes, which provides a great deal of validity to the concept of optical cooling enzymes being possible and, with higher quality laser cooling materials, maybe even applicable to biochemical techniques requiring thermocycling.

2.3.5 Heat Transfer Analysis

A small amount of heat transfer analysis of the previously presented system is appropriate to round out this chapter, especially with the information the rate experiment results provide information about temperature gradients at the surface of our YLF crystal. As previously discussed, we model our YLF crystal as a cuboid as has been done in work performed by the Pauzauskie group before,[82] to avoid complications from the tetragonal bipyramidal geometry while also maintaining similar size and aspect ratio measurements. This allows us to use the Cartesian form of the energy equation to describe the system:

$$\rho \hat{c} \frac{\partial T}{\partial t} = \kappa \left(\frac{\partial^2 T}{\partial x^2} + \frac{\partial^2 T}{\partial y^2} + \frac{\partial^2 T}{\partial z^2} \right) \pm \dot{Q}''' \quad (2.2)$$

where ρ is the density of the crystal (kg/m^3), $\hat{c}$ is the heat capacity ($\text{J}/(\text{kg}\cdot\text{K})$), κ is the thermal conductivity ($\text{W}/(\text{m}\cdot\text{K})$), and $\dot{Q}'''$ is the rate of heat generation or depletion per unit volume in the crystal ($\text{J}/(\text{m}^3 \cdot \text{s})$). I have written the source term as possibly being positive or negative for the HRPYLF system depending on if the input laser will cool or heat

the crystal in question. The source term can also be defined further by:

$$\dot{Q}'''(x, y, z) = \frac{1}{2}\sigma(\mathbf{E} \cdot \mathbf{E}^*) = \frac{2\pi nk}{\lambda\mu c}(\mathbf{E} \cdot \mathbf{E}^*) \quad (2.3)$$

where σ is the optical conductivity of the material, $\mathbf{E}$ is the electric field vector of the laser, $\mathbf{E}^*$ is the complex conjugate of that electric field vector, n is the real part of the refractive index of the crystals, k is the imaginary part of the refractive index of the crystals, λ is the input wavelength in vacuum, μ is the magnetic permeability, and c is the speed of light in vacuum. The electric fields in this equation can be modeled using a software such as DDSCAT.

At all six faces of our cuboid, it is assumed that conduction is the only form of heat transfer that can appreciably occur. All radiative heat transfer is assumed to be negligible due to the small size and relatively low temperatures reached by the YLF crystal.[82] Convection is also near impossible at the face that is in contact with the glass, as there is assumed to be no fluid layer between the crystal and the glass. At the other five faces, the crystal is in contact with a stagnant fluid and thus convection is likely negligible compared to conduction, especially at the relatively small temperature differences the YLF crystal is inducing and the overall volume of surrounding water unaffected by the relatively tiny crystal. For free convection, the heat transfer coefficient of water is between 50 and 3000 W/(m²·K).[85] Using the heat transfer coefficient, the Nusselt and Biot numbers of our system can be calculated to give information about the proportion of conductive versus convective heat transfer and temperature gradients within our solid respectively. The Nusselt number is calculated using

$$Nu = \frac{hL}{k_f} \quad (2.4)$$

where h is the heat transfer coefficient of the fluid, L is the characteristic length of the system of interest, and k_f is the thermal conductivity of the fluid. Even using the highest value of

the heat transfer coefficient in this range yields a Nusselt number of less than 1, implying that convection has very little impact on our system. Use of this estimate also allows us to calculate the Biot number for the system using the thermal conductivity of YLF, which is 6 W/(m·K),[86] using the following equation

$$Bi = \frac{hL}{k_s} \quad (2.5)$$

where h and L are the same as in equation 2.4, but the k_s is the thermal conductivity of the solid rather than the fluid. This yields a value of 0.00125, which informs us that our system does not contain temperature gradients that we need to take in to account. This all aligns with the physical reality of the system, a relatively small crystal dispersed in a relatively large volume of water at room temperature, sitting atop an effective heat sink (the glass bottom of the slide).

Therefore, the boundary conditions at $t = 0$ for each of our faces for conduction is one of constant temperature due to the body of water and the glass slide surrounding the crystal being so large relative to the crystal. The glass and water are both assumed to be at room temperature or 25 °C. Conduction can then occur from the surface of the crystal to heat the enzymes that are in almost direct contact with our crystal of interest. In the case of cooling, thermal energy from the water conduction would be transferred into the crystal, which could then be used by the crystal for additional optical refrigeration, cooling the enzymes at the direct surface. Based on the changes in enzymatic rate activity shown in Figure 2.7e, the calibrated temperature difference value for the crystal used to obtain the enzymatic rate activity data, and the known changes in enzymatic rate with temperature from the temperature controlled UV-vis data, we achieve cooling of roughly 3 °C and heating of approximately 5 °C at the direct surface of the crystal once they have achieved steady state heating or cooling, which is the state they are in during enzymatic rate data collection.

With all of this information about the boundary conditions, our assumptions, and the experimentally determined temperature of the crystal surface, it would be possible to create an approximate model for this system using finite element analysis software such as COMSOL, which would provide further insight into the system we have presented here.

2.4 Conclusions

In conclusion, this work has demonstrated the potential for use of ytterbium-doped YLF crystals as a photonic nanoparticle for use in PCR applications. We show that it is possible to covalently bind enzymes of interest to the surface of the YLF crystals, and that the crystals are capable of optical cooling despite this coating. We demonstrate the clear temperature dependence of our chosen test enzyme and that our crystals can cool significantly enough that we should see a change in the activity rate of the enzyme. We show that the enzymes are still active when attached to the crystals and that their Michaelis-Menten parameters are comparable to those of their unbound counterparts. Control experiments show that we are not causing the conversion of Amplex Red to resorufin through any other pathway than through the catalytic oxidation mechanism of HRP itself. Finally, and most importantly, data shows that optically cooled crystals can have an impact on the function of enzymes attached to their surface and the trend can be replicated. Future work will be dedicated to conjugation of other enzymes, such as *Taq* polymerase, to determine if an application to PCR is possible.

Chapter 3

SOLID-STATE LASER REFRIGERATION OF CORE-SHELL POLYSTYRENE MICROSPHERES

3.1 Introduction

As the optics and semiconductor industries have continued to trend toward smaller and smaller devices, the creation and development of microlasers has been a forefront topic in these fields. Microlasers which take advantage of whispering gallery modes (WGMs) have been of particular interest due to their high Q factors,[87] low lasing thresholds,[88] and their small volumetric footprints,[89] opening up a wide variety of desirable applications for these lasers. WGM lasers have been highly sought after for biological sensing,[90, 91, 92] including intracellular in vivo biosensing[93, 94] and bioresponsive, tunable wavelength sensing,[95] nonlinear optics,[87, 96, 97] and integration onto computer chips or other substrates.[98, 99] Although these types of microlasers show such substantial promise, one remaining challenge is the impact of photothermal heating on these devices and its limit upon the output power WGM microlasers can achieve. When irradiated by a continuous wave source, heating of the microlaser's gain medium can cause greater optical losses, increase the lasing threshold, generate optical effects such as thermal lensing and mode instability, and even photothermally damage or melt the gain medium itself.[89, 100] Photothermal heating also limits the applicability of these lasers in biological applications, as cell death or tissue damage can occur when using high input powers to induce lasing. Some attempts to address the photothermal heating issue have been made, including changing the upconversion mechanism to a looping mechanism that requires less energy[89] and by using pulsed lasers rather than continuous

wavelength ones.[101]

Many designs for WGM microlasers involve the use of lanthanide-doped upconverting nanoparticles (UCNPs) due to the much more efficient nature of upconversion compared to other nonlinear processes, and microlasers fabricated with UCNPs coupled to WGM resonators have been demonstrated.[88, 89] However, UCNPs are not typically considered for WGM lasers in biological applications due to the traditionally high input powers needed to induce population inversion in the rare earth ions, in spite of the other valuable properties they have for lasing. However, the use of UCNPs in the NIR wavelength range does open the door to utilizing anti-Stokes cooling processes that could help offset any photothermal heating produced via continuous wave irradiation. In short, we could create a radiation balanced microlaser in which the input laser source both induces lasing and removes thermal energy from the system generated by said lasing.

In this chapter's work, following a design previously reported by our group,[102] polystyrene microresonators were coated with UCNPs doped with different concentrations of Yb^{3+} ions in order to determine the UCNPs' ability to cool the microresonators under continuous wave irradiation. Polystyrene beads have another relevant application in biophysics measurements, as they are often used as attachment points for single molecule measurements using an optical trap.[103] There are a number of factors that can impact the linkage between the bead and the molecule over time, including singlet oxygen generation and temperature.[104] Polystyrene is a well known sensitizer for singlet oxygen generation, which limits the time that can be taken for measurement, on top of its likelihood to heat up while being held in the trap. Inclusion of UCNPs at the surface can potentially aid in reducing both of these factors. The temperature factor is of course reduced through optical refrigeration and although singlet oxygen generation at the surface of rare earth doped fluorides is possible,[105] it is less

likely than at a bare polystyrene surface. In fact, UCNPs are more likely to be used as photoactivators for other reactive oxygen species sensitizers than as sensitizers themselves.[106] As will be shown later in this chapter, it was found that the UCNPs cool significantly at the surface of the spheres, and that their cooling significantly reduces the overall temperature of the microresonator compared to uncoated microresonators, showing promising capabilities for the achievement of radiation balanced lasing in a microsphere laser and for biophysics applications.

3.1.1 Sodium Yttrium Fluoride UCNPs

As briefly mentioned in the introduction, NaYF is one of the best and most efficient rare earth-doped NIR-Vis upconversion lattices currently known, though it has not reached its full optical cooling potential due to its nonstoichiometric nature. Even still, it has a wide variety of applications in fields such as anti-counterfeiting, photoactivation, bioimaging and sensing, lasing, and, of greatest relevance to this work, laser refrigeration.[107, 106, 108, 109] This is not only due to its efficient upconversion, but also the wide range of possible dopants allowing for a wide range of possible emission wavelengths for any desired application.[108] I will also note one particular manuscript in which the researchers successfully gave mice night vision through NaYF UCNPs bound to their photoreceptors.[110]

NaYF has two primary phases at ambient conditions: the cubic α phase and the hexagonal β phase. Much like YLF, NaYF can be grown through Czochralski methods to form large single crystals or through thermolytic or solvothermal methods to form micro or nanoparticles.[111, 112] The larger Czochralski-grown crystals are preferable for certain applications, such as laser refrigeration. However, only the α phase can be grown using these methods, and this phase demonstrates significantly less efficient upconversion than its β phase counterpart.[113] As a result, use of the thermolytic and solvothermal methods are

much higher when creating crystals for upconversion applications. NaYF synthesis work in the Pauzauskie group falls under the solvothermal umbrella (that being hydrothermal synthesis, which uses water as the solvent) and the UCNPs made for this work were done via thermolytic synthesis in collaboration with the Molecular Foundry at Lawrence Berkeley National Laboratory. In the thermolytic method, rare earth chlorides or trifluoroacetates are dissolved in organic solvents and heated to the point of thermal decomposition under inert atmosphere, producing the desired highly monodisperse UCNPs.[112]

The exact type of UCNPs used for these experiments are core shell β -NaYF nanoparticles in which the core is doped with various concentrations of ytterbium and the shell is doped with 20% gadolinium, both in place of yttrium. The gadolinium shell is necessary to reduce losses in photoluminescence that can occur at the surface of the Yb^{3+} -doped NaYF core due to lattice strain, surface defects, or any remaining ligand at the surface.[89] These problematic factors are significantly more likely in nanoparticles than micron-sized particles due to their high surface area to volume ratios, meaning reducing these losses as much as possible is critical to getting a useful ratio of upconversion out of the NPs. The $\text{NaY}_{0.8}\text{Gd}_{0.2}\text{F}$ shell has minimal lattice mismatch with the core and is optically inert at the input wavelength, allowing the photons generated by the core Yb^{3+} ions to escape without encountering any surface quenching sites while not contributing any luminescence of its own.

3.2 Materials and Methods

3.2.1 Robot-Assisted Synthesis of Core-Shell NaYF UCNPs

The design for the microspheres tested in this work is shown in Figure 3.1a. The nanocrystals were synthesized by collaborators in the Chan group at the Lawrence Berkeley National Laboratory’s Molecular Foundry in a procedure similar to previously described work.[88,

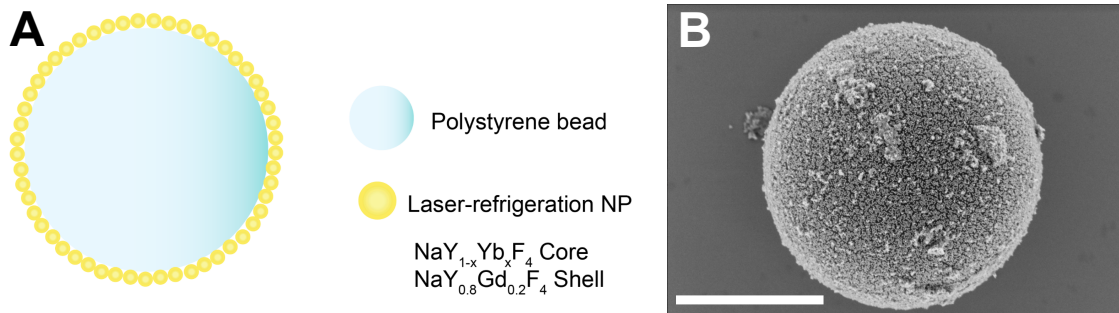


Figure 3.1: (A) Representation of the design of the cooling polystyrene microspheres and (B) SEM image of the Yb³⁺-doped UCNPs coated polystyrene microsphere. Scale bar is 3 μm

89, 113] In brief, the core nanocrystals were synthesized via a thermolytic synthesis method performed via Schlenk line chemistry. This involves dissolving chloride salts of rare earth elements in the desired molar ratio in oleic acid and 1-octadecene and heating to 100 °C under vacuum, creating rare earth oleate complexes. These rare earth oleate solutions are then pumped with vacuum and purged with nitrogen until all water and oxygen are removed. Then sodium oleate and ammonium fluoride are added under nitrogen flow and once again pumped and purged. This mixture is heated to 320 °C to induce thermal decomposition, leaving behind the Yb³⁺-doped NaYF core nanoparticles. These nanocrystals are then shelled with the gadolinium-doped material using a robotic nanocrystal workflow with optimized size and dopant control and high throughput (WANDA). Following an automated procedure, alternate injections of a 20% GdCl₃/YCl₃ solution and a sodium trifluoroacetate solution were introduced to the core solutions every 20 minutes at 280 °C. After cooling, the core shell NaYF UCNPs can be purified and stored in n-hexane until needed.

NaYF₄ UCNPs with multiple different Yb³⁺ concentrations were fabricated using this process, though this work is only concerned with the UCNPs doped with 8% and 10% Yb³⁺ ions. A monolayer of the synthesized UCNPs was deposited onto the surface of PS spheres

5 μm in diameter using a solvent swelling and deswelling process as described previously.² The polystyrene spheres are swelled using an 8% by volume chloroform solution in butanol. The UNCPS are then introduced and the solution is vortexed, then left at room temperature for four hours. The spheres are then washed with ethanol to reduce the swelling, leaving a layer of UNCPS upon the surface. A scanning electron microscopy image of the completed spheres is also shown in Figure 3.1b.

3.2.2 *Mean Fluorescence Measurements*

Mean fluorescence measurements are performed using a sample chamber made up of a glass microscope slide and slide cover, held together by a double-sided adhesive spacer with a thickness of 100 μm . A small aliquot of the sample of interest is dispensed into a well inside of the spacer, holding the sample in place. Prior to any measurements, careful background correction is performed using a sample chamber containing only the solvent that the microspheres will be dispersed in to eliminate any potential fluorescence of the glass under 1020 nm irradiation. The microsphere of interest is placed in the path of the 1020 nm diode beam and optically trapped and held at least 20 μm away from the top or bottom of the chamber. In some cases, the microspheres had adhered to the glass slide cover before trapping could take place, and were observed near to that surface instead. The mirror path is then adjusted to allow the fluorescence signal of the Yb^{3+} ions to the spectrometer via a fiber coupling. The resulting spectrum is then run through a Python script written by a previous member of the research group, which integrates the area underneath the curve of the spectrum to find the expectation value, or mean wavelength value of the spectrum. Multiple measurements are taken using constant laser power that is modulated by the neutral density filter wheel described previously, so that mean wavelength value can be plotted against laser power to observe whether the mean wavelength value redshifts or blueshifts with increasing laser

power.

3.2.3 Calibration of Mean Fluorescence Measurements

The data obtained using the method and code described above will not output a temperature shift value like the cold Brownian motion code is capable of. In order to put our mean wavelength shift in terms of temperature, a separate calibration measurement must be performed on the sample of interest. An aliquot of the microsphere sample of interest dispersed in water is dispensed into the chamber of a temperature-controlled perfusion chamber. The perfusion chamber is then placed on the stage of the inverted microscopy setup similarly to previous samples and hooked up to the temperature controller. Two thermocouples are placed: one inserted into the metal ring of the chamber and one taped down onto the glass surface of the sample area as close as possible to where the mean fluorescence measurement is taking place. This allows us both control over the chamber's temperature while also granting insight about the actual temperature near the sample itself. Mean fluorescence measurements are then taken at a constant laser power (7.6 mW) and at a known temperature, starting at 25 °C and increasing in increments of 2.5 degrees up to a maximum of 45 °C. Mean fluorescence can then be plotted against the temperature measured at the sample area thermocouple, generating a dataset that can be fit with a linear trendline. This trendline can be used to extrapolate temperature change from mean wavelength shift, allowing for more practical analysis of the cooling power of the UCNPs.

3.2.4 Cold Brownian Motion Measurements

A trapping chamber similar to the one described previously is also used for these experiments. These measurements can only be performed on microspheres that are still freely floating in solution, as the Brownian motion of the particles is a necessary part of the measurements.

Single free floating microspheres were located and optically trapped via 1020 nm irradiation at least 20 μm away from the top or bottom of the chamber to reduce any influence of the surface on the microsphere's Brownian motion. After the microsphere is stably trapped, a piezostage is activated to oscillate in the x-direction at a rate of 32 Hz with an amplitude of 1 V. The PSD generated by the motion of the stage and particle is then collected. A diffusion coefficient can be extracted by fitting this data with a Lorentzian fit.

3.2.5 SEM Measurements

SEM Imaging was performed using a ThermoFisher Scientific Apreo SEM in the Molecular Analysis Facility at UW.

3.3 Results and Discussion

As can be seen from the SEM image in Figure 3.1b, the swelling and deswelling process was very effective at depositing a relatively clean and near monolayer of UCNPs across the surface of the polystyrene resonator. The thickness of this layer can be adjusted based on the amount of synthesized UCNPs introduced to the polystyrene spheres during the swelling process and as such can be tuned for optimization in cooling applications. For these applications, it is important to deposit enough crystals to generate a significant amount of cooling power across the whole surface of the resonator, but without building up such a thick layer that we generate quenching effects caused by photon reabsorption in neighboring crystals, retaining heat in the system. By starting with a layer as thin as possible, we can determine if greater than monolayer coverage is needed to induce significant enough cooling for the desired applications and work to thicken the coating if needed.

Mean fluorescence cooling data for both the 8% and 10% Yb^{3+} -doped UCNPs is shown in Figure 3.2. Panel (a) shows a representative spectrum of the signal from the UCNPs,

demonstrating the need for mean fluorescence testing rather than any ratiometric work due to the lack of distinct peaks in the Yb^{3+} ion emission range. Mean fluorescence analysis across several microspheres coated with the 8% dopant concentration show redshifting with increasing laser power in four out of five tested spheres, indicative of laser cooling occurring at a relatively high success rate within the UCNPs. The 10% dopant concentration demonstrated a lower rate of successful cooling, with only two of five resonators demonstrating redshifting with increasing laser power. These differences in cooling capability could be due to inconsistent coating of the resonators, either having too few or too many UCNPs leading to insufficient cooling capacity. As can be seen in the SEM image in Figure 3.1a, the coating is not a perfect monolayer and a resonator with too many additional clumps could be hampered by this. Additionally, a smaller number of 20% dopant samples were tested, but demonstrated significant blueshift across all tested resonators. This is likely due to concentration quenching effects that can happen at higher ytterbium concentrations as neighboring ions are more likely to absorb emitted photons. It was determined that the 8 and 10% dopant concentrations were of more interest for continued testing as a result. Of note is that, of the cooling resonators, these UCNPs demonstrate comparable redshift whether they are trapped or adhered to the glass slide at the bottom of the trapping chamber, meaning the resonator's surroundings do not have a large effect on its cooling behavior.

This mean fluorescence shift is then calibrated to a temperature using the calibration curve shown in Figure 3.2b. The slope of the line generates a $\delta T / \delta \lambda$ dependence that can be used to convert mean fluorescence wavelength shift to temperature change. When this conversion factor is applied to the mean fluorescence data, it can be seen that the 8% doped coated resonators consistently cool close to or upwards of 10 °C, which is a substantial cooling magnitude when taken under aqueous conditions. The 8% doped UCNPs can even cool by as

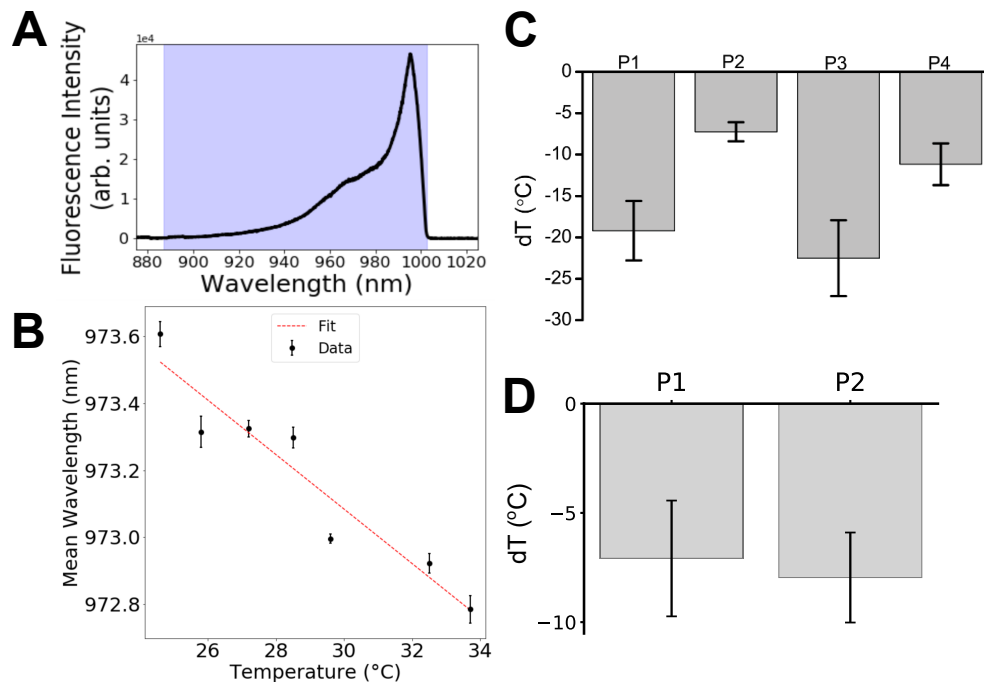


Figure 3.2: Mean fluorescence cooling data from the microspheres. (A) Representative spectrum from the NaYF UCNPs. The blue region is the area of integration. (B) Calibration curve taken from an 8% doped resonator used for temperature calibration of the mean fluorescence data. (C,D) Calibrated temperature shifts of the 8% and 10% doped microresonators that demonstrated cooling trends. Error bars were generated from standard deviation of the original mean fluorescence measurements.

much as 23 °C, which would be able to offset a significant amount of heating in polystyrene microspheres in radiation balanced lasing or biophysics applications. The 10% doped coated resonators cool less, but are more consistent, landing at around 7 °C across the two cooling resonators.

The next thermometry method to apply to the coated microresonators is cold Brownian motion thermometry. This technique gives us a clearer picture of how the UCNPs' cooling capability is affecting the environment directly around the microresonator. Cold Brownian motion data from one of the 8% doped resonators compared to an uncoated polystyrene sphere of identical diameter is shown in Figure 3.3, including the raw voltage trace, PSD, and the processed diffusion coefficient data. As can be seen, regardless of whether the resonators are coated with UCNPs or bare, the diffusion coefficient is increasing and indicative of heating. However, the coated microsphere heats by a much smaller margin than the bare microsphere, reaching a final diffusion coefficient roughly six times lower than the uncoated microsphere. Considering the radius of the resonator is significantly larger than our layer of UCNPs, it is perhaps unsurprising that one thin layer of UCNPs is not enough to offset the amount of heating from such a large body. Even in spite of that fact, this clearly shows that even if the local cooling of the UCNPs is not enough to fully overcome the native heating of the polystyrene, it would allow for the use of much higher input laser powers for lasing or trapping before negative effects begin to show themselves especially when compared with bare microspheres.

It must be noted that the diffusion coefficient values derived from the cold Brownian motion data is in units of V^2/s rather than the more standard m^2/s . This is due, unfortunately, to a limitation of the data collection method that makes calibration of the data from volts to meters impossible due to the size of the microspheres. The microspheres used

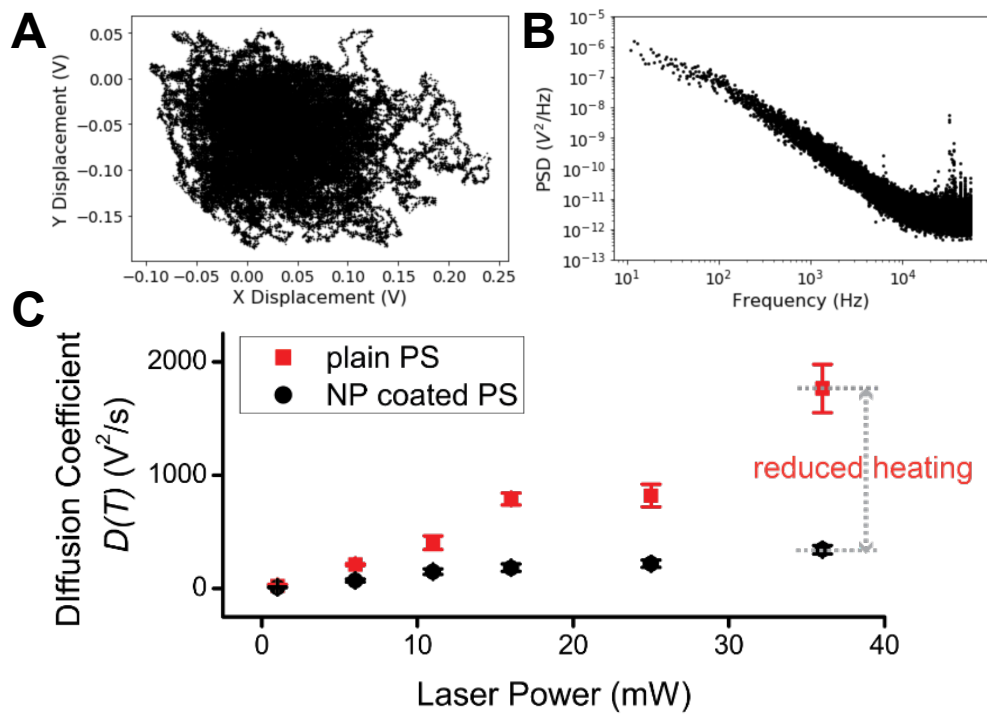


Figure 3.3: Brownian motion data obtained from UCNP coated microspheres. (A) Voltage trace showing the random motion of the particle when held in the optical trap. (B) The PSD spectrum generated from the voltage trace. (C) Comparison between uncoated and UCNP coated polystyrene spheres. The large increase in temperature dependent diffusion coefficient of the uncoated sphere implies much greater heating than the coated microsphere, which only demonstrates a small increase.

in this paper have a diameter of $5\text{ }\mu\text{m}$, which is much larger than the particle size used for these types of measurements in the literature, which is between 500 nm and $1.5\text{ }\mu\text{m}$.^[34] The oscillation amplitude of the piezostage is too small relative to the size of the microsphere to produce the delta-function spike needed for calculation of the calibration constant between volts and meters. The calibration peak appears extremely broad and would not be suitable for calculation of the constant due to the increased area of integration caused by the peak widening. Increasing the oscillation amplitude in an attempt to narrow the peak destabilizes the hold the trapping laser has on the microspheres, causing them to fall out of the potential well and also rendering data collection impossible. As such, the units remain uncalibrated and an exact measurement of temperature change in the environment from this Brownian motion data is not possible. However, we believe that this dataset still demonstrates the stark difference between an uncoated microsphere and a coated microsphere and assists in our conclusions regarding reduced heating. In order to overcome this barrier, heat transfer modeling could be performed in the future using spherical heat transfer theory previously developed by the Pauzauskie group.^[114]

3.4 Conclusions

To conclude, it was found in this work that polystyrene resonators coated with Yb^{3+} -doped NaYF nanocrystals of multiple dopant concentrations are capable of local cooling in the UCNPs at the surface and demonstrate significant reduced heating of their surroundings when compared with uncoated microspheres. The UCNPs were able to locally cool by as much as $23\text{ }^{\circ}\text{C}$ below room temperature over a laser power increase of roughly 35 mW . Understanding the exact quantitative heating of the environment in units of temperature around the microspheres is limited due to problems with the calibration method used to collect the data, but the change in the diffusion coefficient of the microspheres still clearly demonstrates reduced

heating when compared to comparable uncoated spheres. These data points suggest that the coated microresonators have a strong case for potential applications in radiation balanced microlasers or as single molecule biophysics substrates. Reduced photothermal heating will allow for higher input powers into microspheres doped with both cooling and lasing rare earth materials without causing thermal lensing or damage to the gain medium. It is even possible that one wavelength could be used to induce both cooling and lasing, though confirming this possibility will require additional calculations. Reduced photothermal heating and possible reduced generation of singlet oxygen due to the surface coating of polystyrene will also allow for higher energy optical traps to be used for biophysics measurements, possibly opening the door to being able to probe proteins with higher binding affinities than before.

Chapter 4

TEM/STEM IMAGING AND TOMOGRAPHY OF NOVEL FLUORIDE STRUCTURES

4.1 Introduction

Another technique of importance to this dissertation is transmission electron microscopy, or TEM. Although this technique was not applied to the previously discussed projects due to particle size limitations, this technique was used to generate a great deal of publishable data of novel micro- and nanostructures synthesized within the Pauzauskie lab. Much of the information presented in this chapter would not have been possible to collect without use of TEM/scanning TEM imaging, as scanning electron microscopy could not capture the unique interior structures of the crystals of interest. I additionally helped to further develop techniques that previous lab member Dr. Alex Bard had introduced to the group, primarily the collection of tomography datasets used for three dimensional reconstruction of these unique morphologies. These S/TEM experiments were used to guide further experimentation with the syntheses that created the microstructures and the data will feature in upcoming manuscripts describing the unique properties of the materials due to these structures.

4.1.1 Transmission Electron Microscopy

Considering that this is primarily a dissertation about optical refrigeration, a full deep dive into the exact workings of an electron microscope is unnecessary. However, it is still relevant to cover the basics of the instrument's history and function, especially in terms of the specialized techniques used for this data collection. The electron microscope functions through

a very similar process to the optical light microscopes many of us played with as children or used in high school biology to observe the difference between plant cells and animal cells. A light source shines on or through the sample of interest, and a series of lenses magnifies the image so that it can be viewed by the human eye. Magnification is a technique that has been known and used for hundreds of years, but Antonie van Leeuwenhoek is typically credited for popularizing the microscope's use for biological samples all the way back in the 17th century.[115] Improvement in the quality of optical lenses and clever use of multiple lenses in compound microscopes has brought optical microscopy to where it is today. It is a very useful technique for quickly capturing images of structures on the milli- to microscale, but it has a fundamental limitation that it can never truly surpass: the diffraction limit. Light can only be focused down to a point roughly half the size of the wavelength being used, which for visible light places us in the 200-400 nm range. Attempting to image any object or feature smaller than this range with visible light simply will not work (well, you'll get an image, but it will be blurry).

This diffraction limit does carry with it a question: if the diffraction limit is based on wavelength, why not use as short a wavelength of light as possible? The first answer to this is that we do not go as short as possible because that involves using gamma radiation, which is not super compatible with human life and has an unfortunate tendency to penetrate matter, requiring thick shielding with dense materials. X-rays are another option, having wavelengths on the order of picometers, but demonstrate that similar pesky tendency to penetrate matter without being appreciably refracted by said matter, meaning they cannot be focused the same way as visible light. This is a helpful property for macroscale medical imaging, but not for the goal of nanoscale imaging. With options for lower wavelength light exhausted, we turn instead to an electron beam with a wavelength two orders of magnitude smaller than x-rays.

This, of course, generates some additional challenges because electrons are actual particles with mass and thus cannot be refracted by a physical lens, but their charge does allow them to be manipulated, accelerated to incredible speeds (on the order of the speed of light), and focused by an electromagnetic field. Samples must also be very thin and under ultra-high vacuum in order to prevent electron momentum loss in the column due to collisions with environmental gases. Even with these downsides, electron microscopy has become a widely used technique for imaging of all kinds of samples, including biological,[116, 117] and many other techniques can be used in conjunction with imaging to obtain information about crystal planes, elemental data, and full three dimensional models of the imaged sample. It has even become possible with careful spherical aberration correction to resolve individual atoms and the space between them.

Next, I will discuss the specific techniques used for the data collection presented in this chapter. The first of these techniques is electron diffraction, which is a well known technique for gaining information on the local crystal structure of the sample. In this chapter, electron diffraction is primarily used to confirm information about the crystal structures that we already know or at least suspect, and so I will not dive too deeply into the inner workings of it. This technique is based on the physics of diffraction described by Laue and the Braggs in the early 20th century,[118, 119] which tells us that electrons with a wavelength on the order of a crystal lattice spacing will only diffract at certain angles. By measuring these angles, we can determine where atoms are located in our structure and derive a crystal structure from it. We generate the pattern by focusing the image down to a single point in diffraction mode. With the imaging plane no longer taken up by the full image, we can see the diffraction spots generated by the individual crystal in the path of the beam. If multiple crystals are in the selected area, a different type of pattern can be seen. Instead of spots, rings at known

distances are generated by the different orientations of the multiple crystals. This pattern can be radially integrated to generate a pattern similar to those generated by powder x-ray diffraction methods. Diffraction mode can also be used for dark field imaging of the sample in question by selecting a diffraction spot in the pattern using the objective aperture. After returning to the normal imaging mode, the image's brightness is now inverted with the surroundings being dark and the crystal generating the selected diffraction spot appearing bright. The image is now being formed only by the electrons that were diffracted at the angle necessary to pass through the placed objective aperture. This does mean that the images are much dimmer and lower in resolution than typical bright field images, but the technique is nonetheless very useful for observing individual single crystalline domains in clusters present in the sample, or determining if an object is indeed a single crystal.

The next technique to discuss is energy-dispersive x-ray spectroscopy, or EDS. This technique goes by a wide variety of names, but all describe the same process. The electron beam is high enough in energy to excite a ground state electron in a low level orbital shell, leaving a hole behind in its place. An electron from a higher level orbital will then fall to fill this hole, releasing an x-ray equal to the energy difference between those two shells that can be picked up by a detector. These x-rays are characteristic of individual elements, allowing this technique to be used for elemental mapping of a sample. These maps can be used to identify interfaces between two different materials, see compositional changes across an individual crystal, and, of interest for laser cooling applications, determine dopant concentrations of elements of interest. This technique also requires the use of scanning transmission electron microscopy (STEM) because the beam needs to be rastered across the sample to generate data about each visible pixel of the sample. There are a few other elemental analysis techniques available via STEM, but the instrument in the Molecular Analysis Facility only has

an EDS detector, and so that is what we use.

The final technique used in this chapter is STEM tomography, which aids significantly in understanding the three-dimensional structure of the samples we observe in the TEM. Because TEM is, true to its name, a transmission based technique, the images that are output by the microscope are effectively a two-dimensional projection of the three-dimensional object sitting on the holder inside the column. The solution to this involves rotation of the sample or detector, and some complex math that lets us stitch it all together. Tomography is a technique a lot of the general public may not realize that they have definitely heard of, as it forms the basis for computed axial tomography, or a CAT/CT scan. This involves placing the area of medical interest into a large tube containing an x-ray generator and detector that can rotate 360° around the patient while taking hundreds or thousands of images at different tilt angles. The concept is relatively the same for STEM tomography, except the sample is what rotates rather than the detector. With the holder used in these experiments, a tilt range of up to 120° was possible, and this level of tilt is more than enough for the mathematics to fill in the gaps in our tilt series. The many images captured at different tilt angles are then pieced together using something called the inverse Radon transform. The exact mathematics are complex, but the premise is that the forward Radon transform can take an n -dimensional function and then create an $(n-1)$ -dimensional projection of that function. Therefore, in the inverse, it can take an $(n-1)$ -dimensional projection of a function and return an n -dimensional function. As mentioned previously, TEM images are effectively 2D projections of 3D objects, meaning that the inverse Radon transform's clever use of multiple Fourier transforms can help us to generate three-dimensional models from two-dimensional images. These models are highly useful in the case of particles with internal structures that are otherwise difficult to resolve in two dimensions or are masked by other features in the images. This technique has

allowed us a much greater understanding of the unique micro- and nanostructures generated by syntheses in our group and should hopefully continue to do so for years to come after my departure. It is even possible that with the correct instrumentation and carefully applied technique, we could closely probe the exact location of the laser refrigeration defects that drive so much of our research.[120]

4.2 Methods and Materials

4.2.1 Porous α NaYF Synthesis

In a typical hydrothermal synthesis 4 mL of 0.25 M aqueous rare-earth precursor solution with the desired dopant concentration, e.g. 5% Yb³⁺ and 95% Y³⁺, was added to a Teflon liner. While stirring, 5 mL of water containing 1 millimole of sodium dodecyl sulfate was added. After mixing for 10 min, 5 mL of 0.8 M sodium fluoride solution was added and stirred for 30 min. The stir bar was removed, the Teflon liner was closed and transferred into an autoclave. The reaction vessel was placed in a preheated oven at 90 °C and the reaction allowed to proceed for 24 h. After removing the autoclave from the hot oven and naturally cooling to room temperature, the product was isolated by centrifugation, washed three times with 30 mL of water and ethanol, respectively, and dried at 80 °C. Finally, the powder was calcined at 300 °C for 4 h in air to remove surface ligands and adsorbents.

4.2.2 Calcium Fluoride Mandala Structure Synthesis

In a typical hydrothermal synthesis 5 mL of a combined solution of 0.18 M calcium chloride and 0.02 M ytterbium chloride was added to a Teflon liner and stirred for 1hr at room temperature. Then 5 mL of 0.4 M sodium tetrafluoroborate were added to the liner and the resulting solution stirred at room temperature for an additional 1 hr. The stir bar was

removed, the Teflon liner was closed and transferred into an autoclave. This reaction vessel was placed in a preheated oven at 180 °C and the reaction allowed to proceed for 30 hours in. After removing the autoclave from the hot oven and naturally cooling to room temperature, the product was isolated by centrifugation, washed twice with water and twice with ethanol, then dried at 80 C.

4.2.3 TEM Measurements

All measurements were taken using an FEI Tecnai F20 SuperTwin (S)TEM instrument with an accelerating voltage of 200kV housed in the Molecular Analysis Facility at the University of Washington with a Gatan UltraScan CCD camera. TEM samples were prepared by drop casting particles in a dilute suspension of water or ethanol onto 200 mesh lacey or holey carbon coated copper grids, then allowed to dry overnight. STEM imaging was performed on the same instrument with a high-angle annular dark field (HAADF) detector. STEM-EDS measurements were taken using an Elite T EDAX EDS with a dwell time of 100 μ s per pixel and 16 frames of repetition, leading to a scan time of around thirty minutes. Tomography datasets were collected and compiled using the FEI Inspect3D software and visualized using IMOD.[121] The exact tilt series covered a range from -60° to 60° of tilt, the maximum range of the instrument stage. The sample was tilted to 45° in steps of 0.5° and in steps of 3° between 45 and 60°. The same tilt sequence was used for the negative tilts as well.

4.3 Results and Discussion

The first material to be discussed is a unique porous α NaYF structure that was discovered in the Pauzauskie lab as a result of a collaboration project with the Pozzo group. In a recently published manuscript,[122] it was found that inclusion of a surfactant (sodium dodecyl sulfate, SDS) assisted in the creation of almost exclusively the β phase of NaYF

when combined with sonication. It was hypothesized that this concept could also be applicable to the hydrothermal methods employed in the Pauzauskie lab to generate primarily the β phase of NaYF while keeping the overall synthesis temperature lower than is typically needed to induce conversion of the α phase to the β phase. After the first synthesis was completed and characterized with XRD, it became clear that we did not obtain the same phase distribution that was hoped for and the synthesis product was mostly α NaYF. The SEM images collected by fellow lab member Lars Forberger did indicate that there was some shape difference compared to other α phase NaYF grown by similar hydrothermal methods, and so I was asked to take the sample to the TEM.

Initial bright field imaging revealed a fascinating new nanostructure. The crystals showed a clear cubic and crystalline structure visually and using high resolution imaging, but were riddled with pores and almost appeared like tiny sponges. Previous NaYF syntheses had shown no such porosity, as can be seen in comparison to the porous structure in Figure 4.1a. Electron diffraction results shown in Figure 4.1b from single crystals backed up the visual assumption of the expected cubic crystal structure, confirming the structure as α phase NaYF. The diffraction pattern also clearly displays the single-crystalline nature of the cube, generating clear and distinct diffraction spots with no evidence of the ring-like patterns generated by polycrystalline samples. It is also notable that these crystals are much larger than other α phase NaYF crystals I have imaged, with a width of around 200 nm compared to the roughly 50 nm width of non-SDS containing samples prepared by similar hydrothermal methods.

Next, tomography measurements were performed in order to determine the extent to which this porosity existed throughout the full three-dimensional structure of the cube. After some trial and error involved in dialing in the exact parameters to collect the best

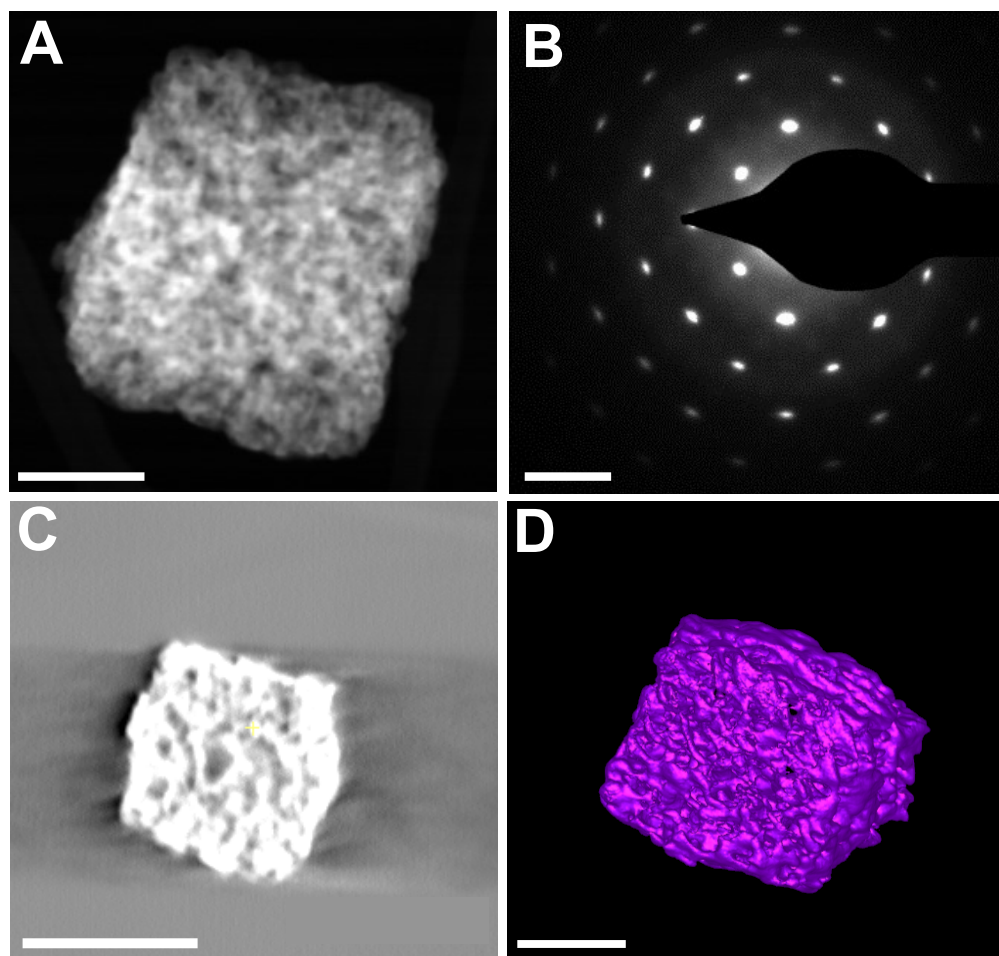


Figure 4.1: TEM Analysis of porous NaYF. (A) HAADF-STEM image of a porous α NaYF cube. Scale bar is 100 nm. (B) Electron diffraction of the crystal shown in A. The pattern shows a clear cubic crystal structure, as is expected of α NaYF. Scale bar is 5 nm^{-1} (C) Tomographic slice of another porous NaYF crystal, showing the open interior structure. Scale bar is 100 nm. (D) Tomographic reconstruction of the crystal in C showing small, continuous pores in the structure. Scale bar is 50 nm.

tomography, a dataset was obtained that can clearly show the interconnected pore network that was established in the sponge-like cubes. A tomographic slice and the reconstructed model are also shown in Figure 4.1. In addition to the slices showing an interconnected network of pores, the reconstructed model demonstrates one particular pore entrance that is continuous throughout the crystal from one side to the other.

With a clearer picture of the internal structure of this material, we can move to hypotheses about the cause of such a unique structure and potential applications for its use in other experiments. We are still not exactly sure what mechanism is inducing the formation of the porous structure, though it clearly has to do with inclusion of the surfactant. One possibility is that micelles formed in solution during the stirring process, as the concentration of SDS in the reaction solution (71.4 mM) is higher than the critical micelle concentration (8.2 mM),^[123] and once crystallization began in the autoclave the initial NaYF phase formed around them. Once the material is cleaned and the SDS removed, the spaces were left behind as empty and generated the final, single-crystalline structure. It is also possible that small crystals form within the micelles and then joined into larger domains via oriented attachment, but this may produce polycrystalline structures rather than the demonstrated single-crystalline NaYF seen in the diffraction data. It is also worth noting that SDS is the only surfactant that has produced this unique structure. Similar surfactants with different carbon chain lengths do not produce the same porous structure but instead form "popcorn" structures described previously.^[124] It is still unclear exactly why SDS is the only surfactant that creates these shapes, but we hypothesize that it has to do with differences in micelle size and critical micelle concentration between the different reagents.

In the interest of my primary dissertation project, the enzyme cooling effort, the additional surface area provided by this structure could be used to bind more enzymes to the

surface than would be possible with non-porous materials. This would mean that each individual crystal would be able to cool more enzymes at once, making for a more efficient enzyme cooling effect. Enzymes are on average roughly 2-7 nm in diameter,[125] meaning that they should be able to slot into some of the pores within the cubes and reaction solution would still be able to reach them for conversion. Of course, this does mean that we will need the structures to cool if we hope to cool enzymes with them. In this way, the porous NaYF demonstrates another unique feature by exhibiting a pressure dependent cooling trend. The crystals show a modest cooling trend at ambient pressure, but begin to heat when placed under vacuum in a cryostat. This could be due to the different media filling in the pores of the sample, with the shift in thermal conductivity or refractive index between air and vacuum essentially creating unique air-NaYF and vacuum-NaYF composite structures that behave differently to each other under 1020 nm irradiation. This means that additional testing will certainly be needed to observe the behavior of these crystals in aqueous environments before they can possibly be applied to enzymatic cooling studies to observe how the incorporation of water into the pores will affect the optical cooling capabilities of the structure.

One last note to make on the topic of these porous NaYF crystals is that although the primary product is the α phase, there are some β phase particles present in a number of the samples I imaged. The β phase of NaYF is the preferable polymorph to produce in a given synthesis as it has a significantly improved upconversion capability compared to the α phase, roughly 4 times better.[113] As such, if this synthesis could be modified to produce β phase NaYF with unique porous nanostructures similar to the α NaYF shown here, it could open up additional unique upconversion applications. Considering that β NaYF grains form through the oriented attachment of mesocrystalline α NaYF structures, it is not outside the realm of possibility that the porous nature of the building blocks would have some effect on the final

structure of the β NaYF grains.[111] However, this does require further experimentation to determine conclusively.

The second material of interest from my TEM studies is Yb³⁺-doped calcium fluoride microparticles. I once again received this sample after SEM imaging had taken place, with that technique revealing what were thought to be relatively unexciting cubic crystals. Upon examination with bright field imaging, I saw much of the same but did notice that the center of the cube appeared to scatter more of the beam than the contrast at the edges would suggest. I then swapped into STEM imaging mode to perform the requested EDS measurements and received the other biggest surprise of my TEM career. This is the only other result that inspired me to leave the TEM room dead zone and make a phone call to confront the scientist responsible, fellow lab member Sankhya Hirani. Much like Lars had been at the porous NaYF discovery, she was just as surprised as I was by the unmistakable circle located in the center of the otherwise cubic crystal, as shown in Figure 4.2. These structures would colloquially come to be known as "mandala structures" in the lab, named after the geometric patterns used in many spiritual traditions that radiate out from a center point (my suggestion of "fried egg" structures was swiftly shot down).

These mandala structures would continue to keep us on our toes, as the EDS scans revealed that the core and shell are made up of different materials. This is not altogether too surprising considering that even in bright field mode I was able to parse that something was odd about the way the crystals' interior appeared to scatter more than the edges. What the EDS reveals is that the ytterbium added to the synthesis, which was intended to be distributed evenly throughout as a 10% dopant, was almost entirely localized within the spherical core. This is in stark contrast to other CaF₂ samples I had imaged in the past, which had the ytterbium distributed more evenly throughout the structure as intended.

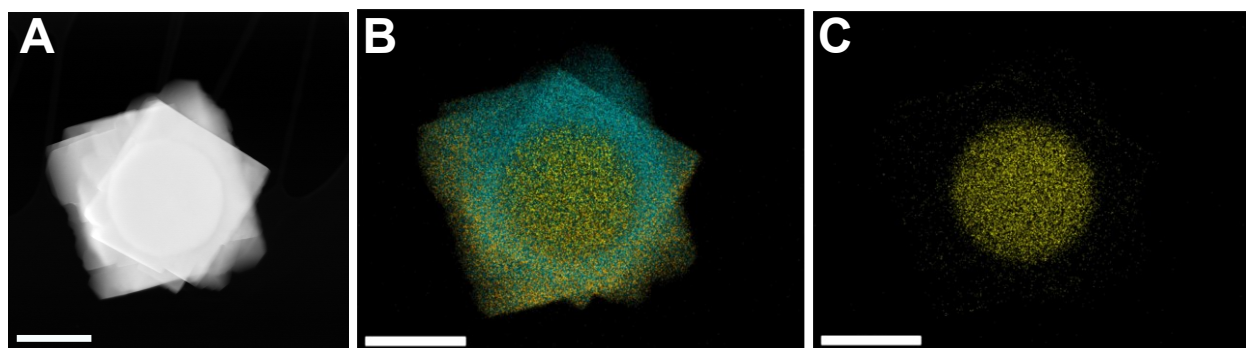


Figure 4.2: STEM-EDS imaging of mandala structures. (A) STEM image of a mandala calcium fluoride structure, showing the circular domain present inside of the cubic exterior. Scale bar is 500 nm (B) EDS map scan results showing that the circular domain also has a different composition from the core. Ca is shown in blue, F is shown in orange, and Yb is shown in yellow. The Yb signal is shown isolated in panel (C), demonstrating its near 100% localization within the circle. Scale bars for B and C are also 500 nm.

The maps also show that the shell is formed out of the intended calcium fluoride, with some fluorine also presenting within the core as well. Elemental maps obtained via EDS are shown in Figure 4.2 As such, our hypothesis is that the core is likely formed out of ytterbium fluoride that formed, nucleated homogeneously, and grew into spheres first. At some point later in the reaction, the calcium fluoride then nucleated heterogeneously and grew in its typical cubic crystal structure around the spheres. This does mean there is some amount of lattice mismatch at this interface, as ytterbium fluoride has an orthorhombic crystal structure and different lattice parameters,[126] possibly introducing some strain into the system that would not be present in the more evenly doped systems.

A second iteration of this synthesis demonstrated a new property: the shell was significantly more susceptible to beam damage. If the imaging TEM beam was held on a crystal for roughly 30 seconds, a portion of the shell would crumble away into the column. The sample could, however, still be imaged without damage in STEM mode and thus EDS and tomography were possible. This fragileness is typically considered a negative property, but

it did allow us to confirm one fact about the crystals that the more robust sample could not. After collecting a STEM image and EDS map of a cluster of particles, I purposefully let the TEM beam damage that same cluster for approximately one minute and then retook the STEM image and EDS map. As can be seen in Figure 4.3, the damage to the shell was enough to loosen the hold the shell had on the ytterbium-rich core and it was pulled away into the column. Considering that most of the shell appears intact, this could imply that there is actually minimal physical contact or bonding between the shell and core if it took so little damage to eject the core. It also shows that the cores are indeed their own separate body from the shell, as the entire core ejected and left behind no trace of ytterbium.

Tomography was also performed on this sample in order to confirm the hypothesis that the inner core is spherical. A tomographic slice and partial reconstruction showing both core and shell is shown in Figure 4.4. The reconstruction shown here only represents the shell section of the crystal, and the empty, roughly spherical region in the center is representative of where the ytterbium-rich core resides. Because the two different materials scatter differently, only one can be effectively represented in the reconstruction at a time and attempting to represent the core generates more artifacts from the inverse Radon transform's estimations. The combination of these pieces of data confirm the hypothesis of a spherical core surrounded by the cubic calcium fluoride structure.

One possible application of these novel mandala structures is for use as cavity resonators, seeing as the core is spherical and could potentially generate whispering gallery modes like the resonators introduced in Chapter 3. The core-shell structure could lead to fewer surface losses, similar to the core-shell UCNPs on the surface of the polystyrene resonators. It would be interesting to see how the optical properties are changed by the fact that so much ytterbium is concentrated in the center of the material, and future work will be dedicated

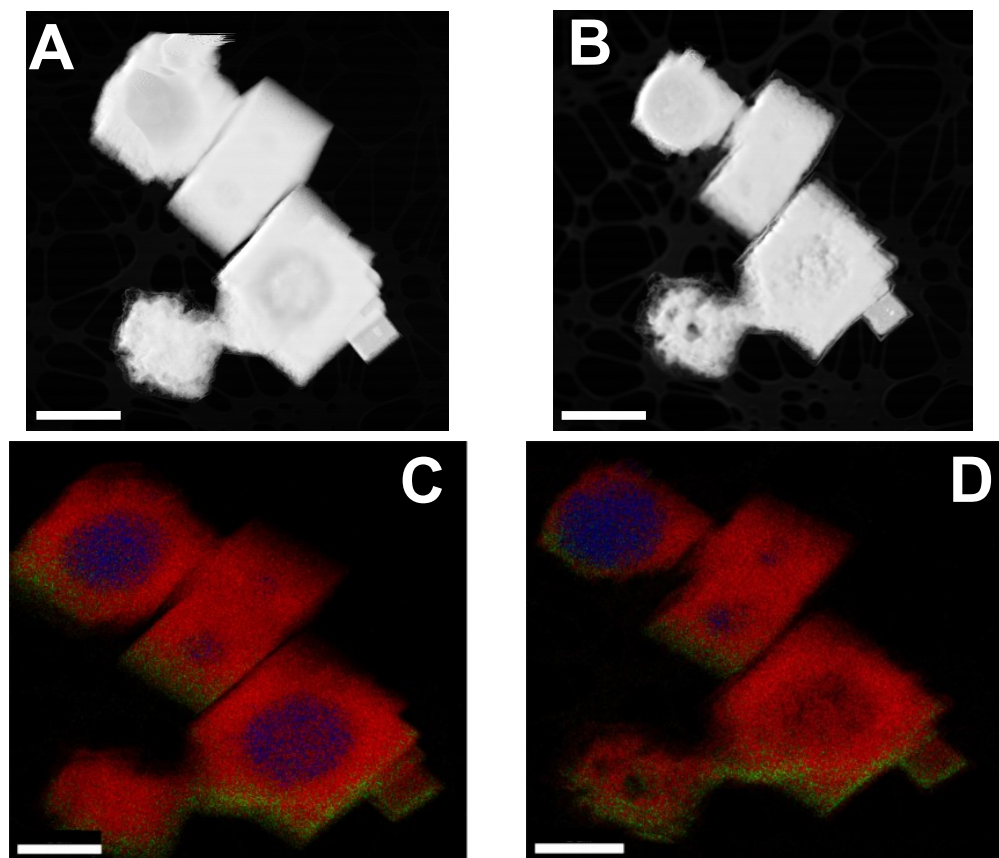


Figure 4.3: STEM-EDS imaging of beam-sensitive mandala structure. Panels (A) and (B) are STEM images of the crystal cluster before and after exposure to damage from the TEM imaging beam. Scale bars are $2\ \mu\text{m}$. Panels (C) and (D) are the STEM-EDS maps obtained from the crystals before and after beam damage. In Panel D, the ytterbium core is no longer present in the bottom right crystal. Scale bars are $2\ \mu\text{m}$. Ca is shown in red, F is shown in green, and Yb is shown in blue.

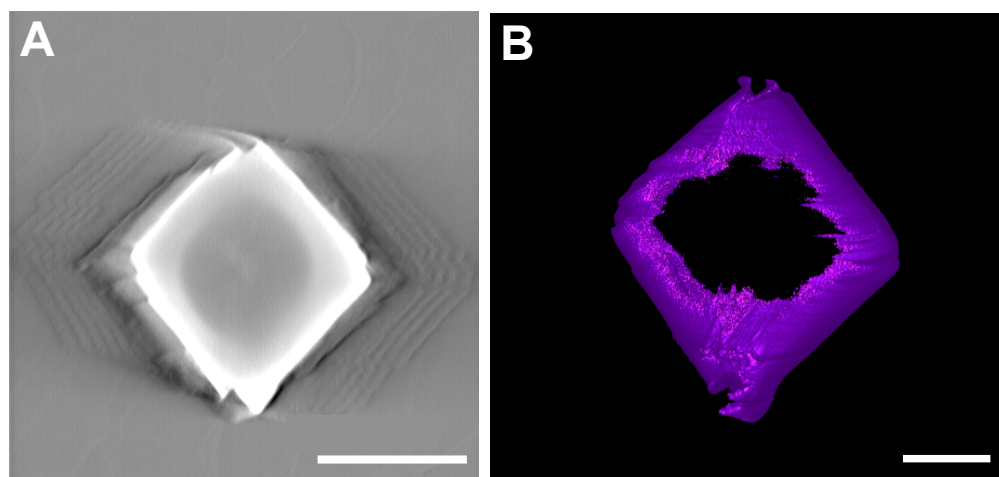


Figure 4.4: Tomographic analysis of mandala structures. (A) A tomographic slice of a calcium fluoride crystal showing that the circular domain within does not appear completely homogenous. Scale bar is 1 μm . (B) Tomographic reconstruction of the shell surrounding the ytterbium-rich core. Only the shell is reconstructed due to the different scattering of the two material types, leaving behind a spherical gap that in reality is occupied by the core, showing its spherical nature. Scale bar is 500 nm.

to testing these hypotheses.

4.4 Conclusions

TEM and STEM data of two rare-earth doped fluoride structures with novel and unique micro- and nanostructures are presented. The methods used for these discoveries are introduced and explained and hypotheses for the formation of the structures are posed. Diffraction, dark field imaging, STEM imaging, EDS mapping, and tomographic reconstruction data are used to gain insight into the interior crystal structures of these samples that would not be possible with other microscopy techniques. Possible applications for these crystals in the context of the earlier chapters of this dissertation are also addressed. Future directions for characterization of these crystals could include liquid cell TEM, which has been used previously by this group to observe crystal growth mechanisms in NaYF before and could

provide insight into the way SDS changes the crystallization pathway.[111]

Chapter 5

CONCLUSIONS

It is difficult to describe all of the work I've done over the last five and a half years in the Pauzauskie group in just a few short paragraphs. In my time here, I have developed a coating synthesis that allows enzymes to bind to the surface of hydrothermally grown YLF crystals, confirmed that these enzymes are still active and can convert substrate comparably to unbound counterparts with similar activity levels, removed doubt that substrate conversion is caused by any non-YLF bound HRP sources, and demonstrated that it is possible to optically influence the behavior of enzymes via laser refrigeration. Enzymes can be slowed down through optical refrigeration, and it could potentially impact the field of biochemistry with some additional fine tuning. And all of this was just on one project.

On other projects, I showed that polystyrene microspheres coated with UCNPs can experience some degree of local cooling as well. When compared to their uncoated counterparts, they heat up their local environment significantly less as well, showing strong possible applications for radiation balanced WGM microlasers and for biophysics measurements. Detailed S/TEM imaging, EDS data collection, and tomographic reconstructions were used to discover unique nano- and microstructures within fluoride crystals and the obtained data will be shown in multiple upcoming publications. These structures could also have applications as to the enzyme and WGM laser projects, with the porous NaYF providing high surface areas upon which enzymes could be conjugated and the mandala CaF_2 structures having spherical ytterbium-doped cores.

Possible future directions include the application of optical refrigeration of enzymes to useful biochemical techniques, such as PCR. The microresonator project lives on as a user proposal with the Lawrence Berkeley National Lab's Molecular Foundry, synthesizing new resonators made from spherical calcium fluoride microparticles that can be doped with rare-earth atoms not just at the surface. Optical characterization of the porous NaYF and mandala structures are ongoing and have already shown results demonstrating unique properties compared to more traditional versions of those materials.

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Appendix A

SUPPORTING INFORMATION FOR CHAPTER 2

Table A.1: Calibrated Cooling Data for HPRYL F Crystals

Crystal no.	Wavelength Shift (Final - Initial) nm	Heating/ Cooling?	ΔT ($^{\circ}\text{C}$)
1	0.09611738861	Cooling	-4.979850456
2	0.1523990481	Cooling	-7.589082556
3	0.005717513707	Cooling	-0.2992058351
4	-0.002191626415	Heating	0.1135483958
5	0.02947340413	Cooling	-1.525154531
6	-0.009237037799	Heating	0.4823495393
7	-0.00618557591	Cooling	0.3204754952
8	0.0168794975	Cooling	-0.9199096754
9	-0.0677787809	Heating	3.512009067
10	0.00726000926	Cooling	-0.3761741716
11	0.01845716661	Cooling	-0.9562674465
12	0.005866262645	Heating	0.3038218283
13	-0.02498962099	Cooling	1.294718713
14	-0.03587607638	Heating	1.858724713

15	-0.04256868437	Heating	2.204812547
16	0.2102922763	Cooling	-10.8925167
17	0.03045076318	Cooling	-1.577656753
18	0.5571807582	Cooling	-28.86757451
19	2.2900664974	Cooling	-15.028377109
20	0.2979483195	Cooling	-15.43627898
21	0.07520900813	Cooling	3.891865511
22	-0.4165195327	Heating	21.57991141
23	0.05222700246	Cooling	-2.705566041
24	0.0127827911	Cooling	-1.054348458
25	-0.04856809742	Heating	2.517500573

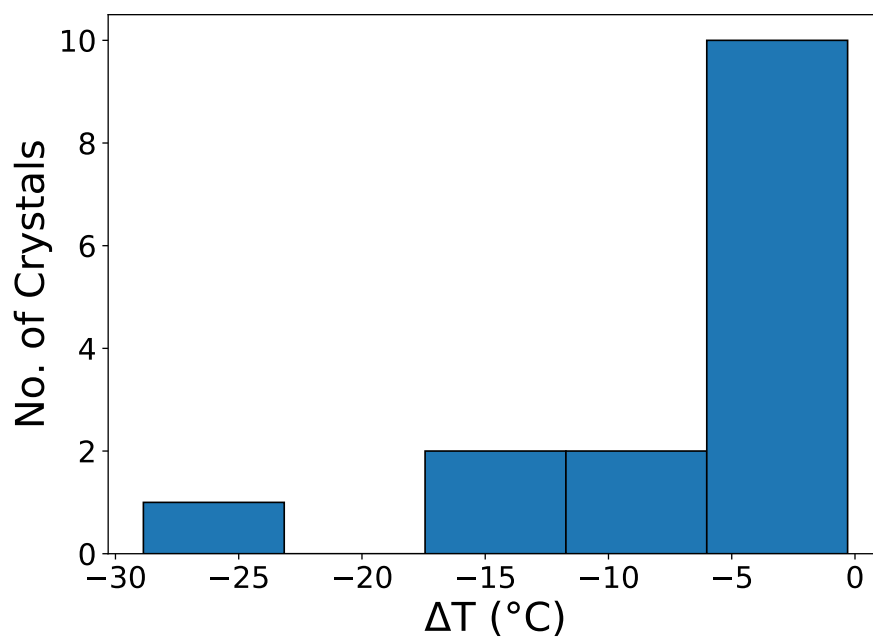


Figure A.1: Histogram of the cooling crystals from Table A.1

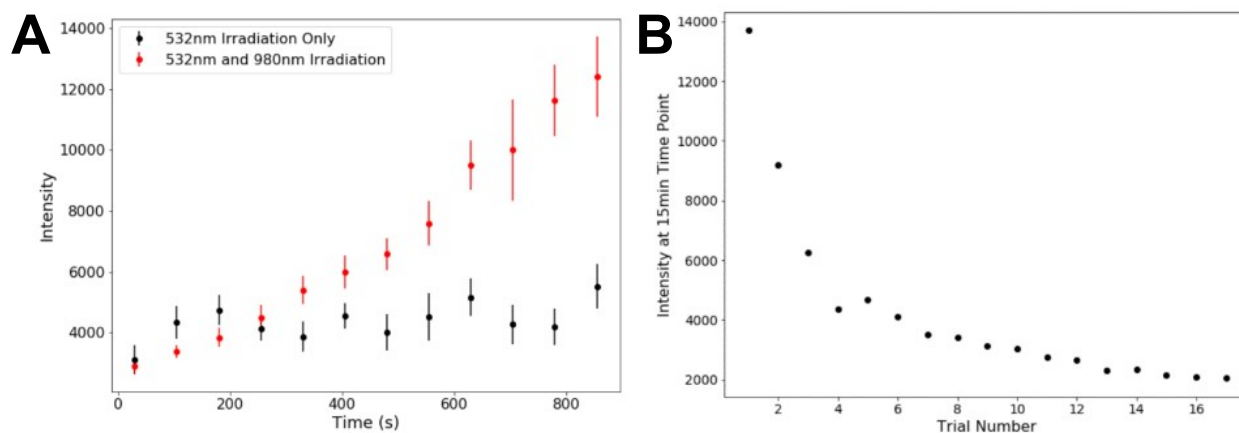


Figure A.2: (A) Data that appears to show a strong heating trend from the HRPYLF crystals before data collection conditions were alternated. (B) Data showing intense degradation of the enzymes over successive trials due to photothermal damage.

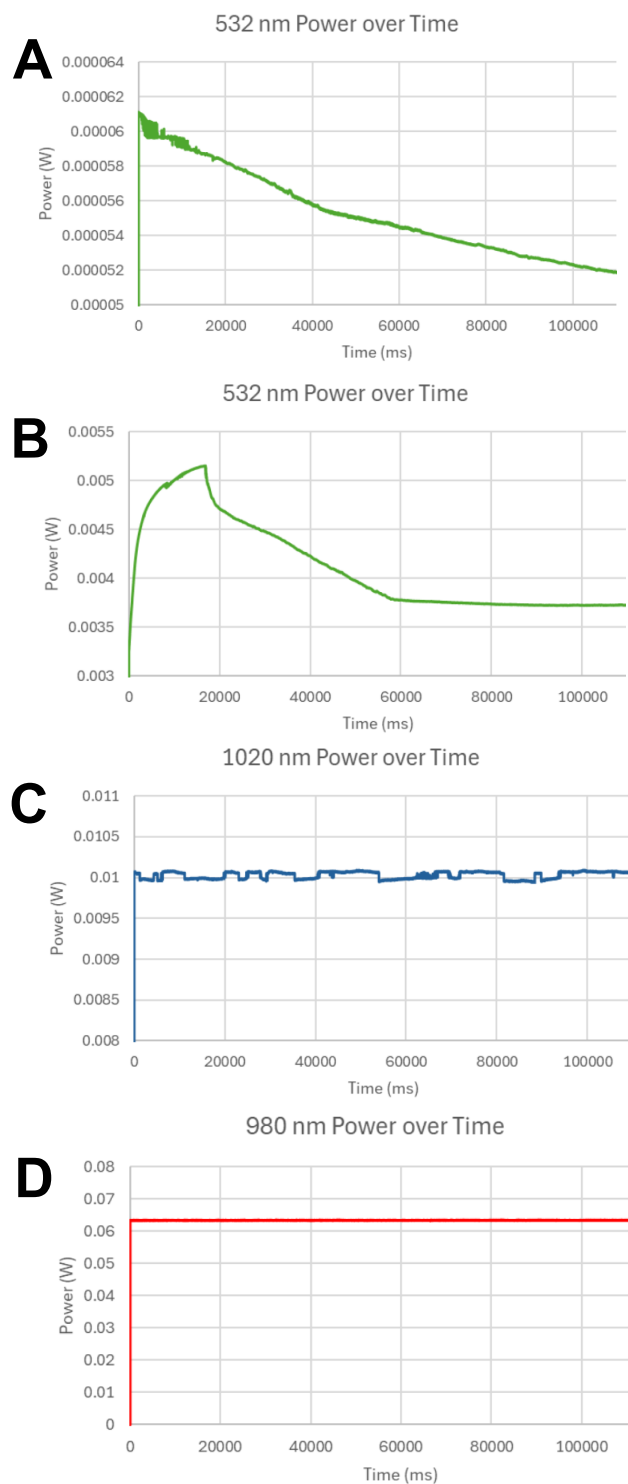


Figure A.3: Testing of the three different lasers for stability. (A) and (B) Stability of the 532 nm laser used for initial measurements over time, taken directly before the objective and directly ahead of the diode respectively. Panels (C) and (D) show the stability of the 1020 and 980 nm lasers respectively.

Appendix B

LIST OF PUBLICATIONS AND PRESENTATIONS

- **R. Gariepy**, A. J. Nuhaily, Z. Feng, and P. J. Pauzauskie. “Optical Refrigeration of Enzymes Attached to Rare Earth-doped Fluorides” (*in preparation* 2025).
- X. Xia*, **R. Gariepy***, R. G. Felsted, A. Teitelbom, E. Chan, and P. J. Pauzauskie. “Solid-state laser refrigeration of core-shell polystyrene microspheres” (*in preparation* 2025).
- L. Forberger, **R. Gariepy**, A. B. Bard, M. A. Hilyard, Z. Feng, S. Hirani, and P. J. Pauzauskie. “Porous α -NaYF – A Pressure Dependent Laser Refrigeration and Heating Material” (*in preparation* 2025).
- E.A. Dobretsova; A. Pant; X. Xia; **R.E. Gariepy**; P.J. Pauzauskie. Safe and Scalable Polyethylene Glycol-Assisted Hydrothermal Synthesis and Laser Cooling of 10%Yb³⁺:LiLuF₄ Crystals. Appl. Sci. 2022, 12, 774. <https://doi.org/10.3390/app12020774>
- M. B. Lim, A. S. Ganas, J. L. Hanson, X. Zhou, B. Hellner, S. Manandhar, **R. E. Gariepy**, F. Baneyx, D. S. Wilbur, P. J. Pauzauskie. Crystalline loading of lipophilic Coenzyme Q10 pharmaceuticals within conjugated carbon aerogel derivatives. Carbon. Volume 164, 2020, Pages 451-458, <https://doi.org/10.1016/j.carbon.2020.02.028>

- **R. Gariepy**, X. Xia, R. G. Felsted, A. Teitelbom, E. Chan, P. J. Pauzauskie. Solid-state laser refrigeration of core-shell polystyrene microspheres. Proceedings Volume 12198, Optical Trapping and Optical Micromanipulation XIX; 121980A (2022). Presented at SPIE.
- **R. Gariepy**, A. Bard, P. J. Pauzauskie. Optical Refrigeration of Enzyme-Coated Rare-Earth-Doped Fluorides. Presented at Optica Biophotonics Conference in April 2023.