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Probing Molecules in Confined Space

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Probing Molecules in Confined Space

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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I would like to dedicate this dissertation to Timothy Lu Vetromile, my mother-in-law and guardian angel. We miss you dearly, but know you are smiling down on us.
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This achievement would not have been possible without some very important people in my life, to whom I am forever grateful. First, I would like to thank my Major Professor, Dr. Randy Larsen, for accepting me into his lab and providing a scientific environment that was both challenging and inspiring. I would also like to thank my committee members, Dr. Brian Space, Dr. Peter Zhang, and Dr. Venkat Bhethanabotla for their scientific insight and direction throughout these years. To my functional yet dysfunctional lab family and fellow Larsenites Christ Whittington, Audrey Mokdad, Meagan Small, Tarah Word and Billy Maza thank you so much for all of the help, laughter and encouragement you have provided over the years.

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Abstract

Despite the plethora of information regarding cellular crowding and its importance on modulating protein function the effects of confinement on biological molecules are often overlooked when investigating their physiological function. Recently however, the encapsulation of biomolecules in solid state matrices (Nafion\textsuperscript{TM}, sol-gels, zirconium phosphate, etc.) has increased in importance as a method for examining protein conformation and dynamics in confined space as well as novel applications in biotechnology. Biotechnological applications include, but are not limited to, bioremediation, biosensors, biocatalysts, etc. In order to better utilize solid state materials as substrates for biological molecules an understanding of the effects of encapsulation on the detailed dynamics associated with physiological function is required as well as a complete characterization of the physical properties associated with the space in which the biological molecule is to be confined. The focus of this research is to probe the effects of confinement on the thermodynamics of ligand photo-release/rebinding to the prototypical heme protein, myoglobin, encapsulated within sol-gel glasses utilizing photoacoustic calorimetry (PAC) and photothermal beam deflection (PBD). Optical spectroscopies (including optical absorption and fluorescence) have also been employed to characterize the molecular environments of materials including Zr-phosphate and metal organic polyhedral (MOPs), thought to be good candidates for novel bio-hybrid materials. The assembly mechanisms associated with MOPs were also examined in order to develop a foundation through which new, bio-compatible MOPs can be designed. Overall the results presented here represent a technological
breakthrough in the application of fast calorimetry to the study of proteins in confined space. This will allow for the first time the acquisition of detailed thermodynamic maps associated with the well-choreographed biomolecular dynamics in confined environments.
Chapter 1: Introduction

1.1 Introduction

The importance of local environment in modulating protein dynamics and ultimately protein function has been well established over the past forty years. What is known is that the cell is a very crowded environment with the presence of up to 400 mg/ml of macromolecules (20-30% of the total volume), and that biological molecules such as proteins have evolved over millions of years to optimally function in this very dense environment.\textsuperscript{1-4} Molecular crowding or the excluded volume effect has substantial influence on the thermodynamics and kinetics of protein processes which eventually propagate to overall cellular function. Diffusion coefficients can decrease 10-fold for both large and small molecules under cellular conditions, thus significantly slowing the rates of diffusional processes. Conversely, processes that involve a decrease in excluded volume (i.e. macromolecular associations, protein folding) equilibrium constants may increase by two to three orders of magnitude depending on the size of the molecules involved. Enhanced equilibrium constants arise from the fact that the most thermodynamically favored configuration is the one in which additional excluded volume becomes available for the other macromolecules.\textsuperscript{1-4}

Yet, even with the knowledge of typical \textit{in vivo} environments and their effects on biological function the majority of \textit{in vitro} protein structure-function investigations are done in bulk solvent systems\textsuperscript{1-4}. A general technique used to examine the effects of crowding on biological macromolecules is the addition of high concentrations of
macromolecules, which were designed to mimic cellular crowding, to solution samples. Most commonly utilized crowding agents include ovalbumin, and bovine serum albumin, as well as synthetic agents including Ficolls, dextrans, polyethylene glycol and polyvinyl alcohol.\textsuperscript{3,4} However, more recently the encapsulation of biomolecules in solid state matrices (Nafion\textsuperscript{TM}, sol-gels, zirconium phosphate, etc.) has become another method to study proteins in confined space.\textsuperscript{3-13} While both crowding agents and solid platforms allow the use of optical spectroscopies to probe protein activity, there are several advantages to employing solid matrices for these types of investigations. For instance, unlike most crowding agents, the encapsulation of proteins in silica based glasses can ensure the inability for protein aggregation due to the templating effect. In addition, solid state materials are chemically and thermally robust providing the opportunity to probe the effects of stress (i.e. temperature, pH, etc.) on confined proteins without altering the solid matrix.\textsuperscript{3,4}

Aside from investigating biomolecules in solid state matrices to mimic physiological conditions, it is also of interest to many biotechnological applications including bioremediation, biosensors, biocatalysts, etc. It is extremely advantageous to use evolutionarily designed biomolecules (enzymes, RNA, DNA proteins polypeptides, etc) as sensors, catalysts, and recognition systems, which requires the ability to trap active stable biomolecules in a solid medium allowing for mobilization and separation from reaction byproducts\textsuperscript{6-22}. Horseradish peroxidase encapsulated in sol-gels (HRPsol-gel) is one example of an extensively studied biocomposite catalyst.\textsuperscript{6,15-18} HRP is capable of oxidizing a plethora of molecules using \(\text{H}_2\text{O}_2\), through the generation of a highly catalytic oxoferryl protoporphyrin IX active site. Smith et al. have shown that encapsulation does not alter enzyme activity, and considerable recoverability is obtained with up to 10 turnovers observed before a decrease in enzyme activity.\textsuperscript{17} HRP is just
one of an array of biomolecules that have already been encapsulated in solid state materials to be employed as optical sensors, catalysts, etc. Accordingly, the improvement of this technology also necessitates knowledge regarding the effect of encapsulation on protein function, as well as, further characterization of potential biomimetic/bioactive materials.

For these reasons, in recent years there has been increased interest in unraveling the effects of confinement on protein activity (ie, ligand binding, electron transfer, monooxygenation, folding/unfolding, etc.).\(^1\)\(^-\)\(^{22}\) The conformational dynamics associated with ligand release/binding to heme proteins (HRP, myoglobin, hemoglobin, etc.) are common systems used for probing the effects of confined space on activity, due to the ease of ligand photocleavage, and the inherent spectral properties of the heme moiety that can be monitored\(^15\). Encapsulation has shown to help resolve solvent slaved intermediates, slow down kinetic events enough to reveal relevant intermediates that are otherwise too fast to observe in solution, and increase protein stability under very harsh conditions.\(^9\)\(^-\)\(^{14}\),\(^{17}\),\(^{18}\) However, accompanying thermodynamic profiles of biomolecular function are lacking.

Traditionally, the energetics and structural changes associated with fast time scale intermediates along a reaction pathway are determined by monitoring the reaction rates as a function of temperature or pressure and analyzing the data using:

\[
\ln\left(\frac{k_{\text{obs}}}{k_b T}\right) = \frac{-\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{R} \tag{1}
\]

(where \(k_b\) is Boltzmann’s constant, \(h\) is Planck’s constant, \(k_{\text{obs}}\) is the observed rate constant, and \(T\) is the absolute temperature) and:
\[-RT\frac{\partial \ln(k_{obs})}{\partial P}_T = \Delta V^\ddagger\] (2)

where $R$ is the universal gas constant, $T$ is the temperature and $P$ is the applied pressure. The activation enthalpy is the given by the slope of equation 1 and the interception gives the activation of entropy. Similarly, the activation volume is equal to the slope of equation 2. In order to construct a complete thermodynamic profile the forward and back reaction rates for all events in a reaction pathway must be determined as a function of temperature and pressure. Unfortunately it is not always possible to measure both the forward and back rates of fast processes. However, a complete thermodynamic profile can be constructed with the knowledge of $\Delta H^\ddagger/\Delta V^\ddagger$ in one direction in addition to $\Delta H/\Delta V$ between transient species along the reaction coordinate.

In contrast steady-state techniques used to determine equilibrium constants can provide knowledge on equilibrium thermodynamic parameters using the van’t Hoff equation:

\[\frac{\partial (\ln K)}{\partial (1/T)} = -\Delta H^o/R\] (3)
Where $K$ is the ligand association constant, $T$ the temperature, $R$ the gas constant and $\Delta H^\circ$ is the reaction enthalpy. Likewise the pressure dependence of an equilibrium constant a reaction can provide the molar volume change by:

$$\frac{d(LnK)}{dP} = \frac{\Delta V^\circ}{RT}$$

(4)

where $P$ is pressure.

Biological processes involve conformational transitions typically on fast time scales (<1ms) making it especially difficult to obtain complete thermodynamic profiles. In addition the reaction mechanisms of biomolecules are usually favored in the forward direction (i.e. $k_f >> k_r$) thus providing another challenge in determining necessary rate constants for the reverse steps. Methods commonly used to probe protein structure/dynamics including high resolution NMR and protein crystallography are limited by their time resolution to access kinetic information for fast events under physiological conditions. While time resolved optical spectroscopies including absorption, fluorescence and circular dichroism can provide kinetic information on sub picosecond to second timescales, they are limited to probing local dynamics related to a specific chromophore. Overall, these techniques cannot directly measure enthalpy and molar volume changes for biological process on fast time scales.

Time-resolved photothermal methods such as photoacoustic calorimetry (PAC), photothermal beam deflection (PBD), transient grating (TG), and thermal lensing (TrL), have been established as powerful techniques for probing the magnitude and time scales of enthalpy and molar volume changes associated with protein function.$^{26-36}$
In general all of these techniques arise from photoexcitation of a molecule in solution resulting in a transition from its ground state ($S_0$) to some higher excited electronic state ($S_n$) following Fermi’s Golden Rule.\textsuperscript{29,37} Deactivation of this state may occur through emission of a photon (fluorescence), non-radiative relaxation, and intersystem crossing (triplet state formation) (figure 1.2). Photoexcitation may also initiate chemical processes including bond breakage/formation, electron transfer, etc. as well as changes in molecular conformation (e.g. cis-trans isomerization) and intermolecular charge redistribution (e.g. change in dipole) resulting in changes in solvent density (also discussed in Chapter 2). If the sample in question undergoes non-radiative decay and photochemical processes then excitation of the sample induces rapid density changes due to both thermal heating to the surrounding solvent and solvent-molecule interactions. The differences among the techniques originate from how this process is detected and analyzed.

PAC is the most straightforward method with the detection of pressure waves (acoustic wave) generated by the rapid thermal expansion and molar volume change of the solvent upon photoexcitation by a pressure sensitive detector (piezoelectric crystal).
Enthalpy and molar volume changes can be determined through the temperature dependence of the acoustic signal and a colorimetric reference (the method is explained in detail in chapter 2). The time range for PAC is typically limited by the time it takes for the acoustic wave to travel across the excitation beam ~50ns to the damping of the acoustic signal on the detector ~10μs.

The methods, TrL and PBD, utilize a probe beam to measure the change in refractive index due to thermal heating and molar volume change. For TrL the probe beam is centered in the Gaussian profile of the refractive index change or density. The expansion (or focus) of the probe beam by a photogenerated lens is detected with a photodetector and monitored as a function of time. In contrast, the probe beam for PBD passed through the edge of the lens and the change in refractive index is detected by a deflection in the probe beam on a position sensor. The difficulty in these methods arises from the fact that the change in refractive index also involves a population lens (absorbance of the probe beam by photogenerated species) making it difficult to separate out the contributions from heat released, and change in molar volume. However, for PBD the population lens is avoided by choosing a probe beam wavelength far from any anticipated absorbing species and enthalpy and volume changes are calculated from the temperature dependence of the probe deflection (discussed in chapter 2). In contrast, for TrL the decay rates of the thermal lens and population lens requires knowledge of the thermal diffusivity and molecular diffusion coefficients to obtain ΔV and ΔH for a reaction. The time scales for these techniques range from generation of the thermal lens, ~10μs, to the thermal diffusion of the solvent ~seconds. However in practice the long timescale is often governed by the quality of the signal and the stability of the probe beam.
Transient grating is also based upon optical detection of a refractive index change by a probe beam. Unlike TrL and PBD, an optical interference pattern between two pump beams generates populations of excited state molecules in a specially modulated profile. The gradient is detected by the probe beam on a photodetector. Similar to TrL and PBD the refractive index change is also multi component. The temporal window is larger than the other methods with detection from femtoseconds to seconds depending on the origin of the grating. In addition, calculation of enthalpy and volume changes can be done under one set of conditions (i.e. temperature). The problem still remains that it is very difficult to separate out the thermal, volume, and absorption contributions to the TG signal.29

To date PAC and PBD have provided thermodynamic insight on nanosecond to millisecond conformational dynamics involved in ligand photolysis to myoglobin, hemoglobin, neuroglobin, as well as, heme protein folding and heme protein model systems, to name only a few.26-36 A majority of PAC and PBD experiments have been performed in solution conditions.26-36 However, a major portion of the research presented here is focused on the use of PAC and PBD to investigate CO release and rebinding to ferrous horse heart myoglobin in confined space and obtain complete thermodynamic profiles associated with ligand binding. To our knowledge, this is the first time they have been employed to probe the effects on energetics and conformational dynamics of biomolecules in confined space. Specifically, do the effects of confinement and consequentially lack of bulk solvent perturb the thermodynamic profiles for the case of ligand binding to hhMb as shown in figure 1.3?
The first solid state material examined in this work is silica based sol-gels, an optically clear porous material known to encapsulate a wide array of biomolecules. Proteins encapsulated in sol-gels have displayed preservation of activity, enhanced stability, enzyme activity and recoverability. In addition, the hydrophilic nature of these materials allows for small molecule diffusion to the captive biomolecules. A commonly used heme-protein system encapsulated in sol-gels for physiological studies is carbon monoxide bound myoglobin, since this protein is water soluble and CO flash photolysis/rebinding can be readily monitored due to its extensively characterized spectroscopic properties, and high yield of CO photocleavage. A detailed summary of CO migration in hhMb can be found in chapter 3 section 3.1. It is also of particular interest for this study since the kinetics and thermodynamics associated with CO

Figure 1.3 Diagram describing the possible change in thermodynamic profiles for CO photolysis from myoglobin in solution and encapsulated in a solid matrix.
photodissociation and rebinding in solution have been previously characterized by PAC and PBD. Thus, this system provides a significant opportunity to explore the use of photothermal methods as a way to unravel, on the molecular basis, the effects of confinement of protein function and ligand migration.

Figure 1.4 Illustrations of potential bio-hybrid materials (left) cytochrome c incorporated metal-organic polyhedral and (right) myoglobin impregnated zirconium phosphate

The two additional systems presented in this work focus on the characterization of materials thought to be good candidates for novel bio-hybrid materials, as well as, systems to study proteins in confined space (shown in figure 1.4). The first, zirconium phosphates (ZrP) are clay like layered materials that are chemically robust, easily synthesized under relatively mild conditions and can serve as good ion exchangers. Although, there are several types of ZrPs based on structural type, the focus here on
αZrP with an interlayer distance of 7.6Å, and expanded ZrP with an interlayer distance of 10.3Å.39-45 Previously, it has been demonstrated that proteins can adsorb in the interlayer space of modified butylammonium exchanged ZrP (BAZrP) with an interlayer distance of 18Å and retain activity, but intercalation has not been established in the unmodified ZrPs.41-44 A better understanding of the adsorption properties and interlayer environment is required to ultimately incorporate biomolecules in unmodified ZrP. To accomplish this, two optically sensitive environmental probes were directly ion exchanged into ZrP and/or absorbed onto ZrP galleries without organic modifications. The photophysical properties, including optical absorption and both steady state and time-resolved emission data we examined to obtain modes of adsorption as well as information on the chemical environment of the bound macrocycles.45

The final systems examined are metal organic materials (MOMs). It was thought that the use of MOMs as porous frameworks for encapsulation of biomolecules could provide not only another solid state matrix to probe the effects of confinement on biomolecules but possibly the development of new biomaterials. One member of the MOM class of structures of interest to biomaterials, the faceted polyhedral also known as nanoballs (NB). These discrete metal organic polyhedral (MOP) are formed through 'one pot' self-assembly and are readily modified by varying the metal and/or the organic ligand to increase cavity size, solubility and functionality.32 The majority of research available on MOPs involves synthesis and design of new structures, however information towards solution structure and stability is limited.36 Before MOPs can serve as platforms for drug delivery systems, and novel protein:MOP complexes a better understanding of solution properties is necessary. The work presented here utilizes the inherent optical properties of (5-OH-bdc)²⁻ and the intact CuOH NB to probe the effects of temperature, pressure, water, pH and ligand exchange on CuOH Nb stability. Here
the photophysical properties of Cu hydroxyl nanoball (CuOH NB) have been utilized to probe CuOH NB stability.\textsuperscript{34} Moreover, the photophysical properties of the intact CuOH NB allow for the first investigation on the mechanism of structure assembly in solution for a MOP.

1.2 References


Chapter 2: Photophysical Methods

2.1 Photothermal Methods

Photoacoustic calorimetry (PAC) and photothermal beam deflection (PBD) have been employed to investigate the magnitudes and lifetimes of enthalpy and molar volume changes associated with many biological systems, including, ligand binding, protein folding/unfolding, electron transfer, and signal transduction, to name only a few \(^1\)\(^-\)\(^12\). In this work PAC and PBD are being utilized to probe the effects of confinement on CO ligand release/binding to myoglobin encapsulated in sol-gel.

A detailed description of PAC/ PBD theory and application as well as instrument set up can be found in previously published work.\(^1\)\(^-\)\(^12\) Both methods originate from the same processes described in figure 1.2 Chapter 1. Photoexcitation of a molecule in solution results in a transition from its ground state (\(S_0\)) to some higher excited electronic state (\(S_n\)) following Fermi’s Golden Rule. Deactivation of this state may occur through emission of a photon (fluorescence), non-radiative relaxation, and intersystem crossing (triplet state formation). Photoexcitation may also initiate chemical processes including bond breakage/formation, electron transfer, etc. as well as change in molecular conformation (e.g. cis-trans isomerization) and intermolecular charge redistribution (e.g. change in dipole) resulting in change in solvent density (Figure 2.1). If the sample in question undergoes non-radiative decay and photochemical processes then excitation of the sample induces rapid density changes due to both thermal heating to the surrounding solvent and solvent molecule interactions. In aqueous solutions this rapid heating is described by:\(^2\)\(^,\)\(^13\)
\( T(r,t) = \frac{2\alpha E_a}{\pi \rho C_p \hbar \omega} \) \hspace{1cm} (1)

Where \( \alpha \) is the absorption coefficient, \( E_a \) is the total energy per pulse, \( \rho \) is the density of the solvent, and \( C_p \) is the specific heat capacity of the solvent.

This change in temperature results in density change which gives rise to both a pressure wave and a change in refractive index. These two effects provide the physical origins for PAC (pressure gradient) and PBD (refractive index) techniques.

2.1.1 Photoacoustic Calorimetry PAC utilizes the fact that this rapid change in temperature induces a change in volume of the illuminated solution that is proportional to the thermal expansion coefficient of the solvent:\textsuperscript{14,15}

\[ \Delta V = \beta \Delta T \] \hspace{1cm} (2)

This volume change generates a change in pressure by:\textsuperscript{2}

\[ \Delta P = -\left( \frac{1}{\kappa_T} \right) (\Delta V/V)_T \] \hspace{1cm} (3)
Where $\kappa_T$ is the isothermal compressibility of the solvent and $\Delta V$ is the volume of the illuminated sample. This newly generated pressure wave gives rise to an acoustic signal that is measured by piezoelectric transducer.

The measured acoustic signal is directly proportional to the heat deposited and/or molar volume changes of the sample upon photoexcitation. The corresponding PAC signal for the sample can then be expressed as:

$$S_s = K E_a (\beta) \left( \frac{1}{\rho C_p} \right) Q + \Delta V_{con}$$

(4)

Where $K$ corresponds to the instrument response parameter, $\beta$ is the coefficient of thermal expansion of the solvent ($K^{-1}$), $\rho$ is the solvent density ($g \text{ mL}^{-1}$), $C_p$ is the solvent heat capacity ($\text{cal g}^{-1} \text{ K}^{-1}$), $Q$ is heat released to the surrounding solvent ($\text{kcal mol}^{-1}$), and $E_a$ is number of Einsteins adsorbed. The change in volume due to all non-thermal relaxations (i.e. conformational changes, chemical processes, electrostatics, etc) is $\Delta V_{con}$ ($\text{mL mol}^{-1}$). The instrument response parameter is unique to each experiment. Thus to eliminate, $K$, a reference compound is utilized in which the electronic excited state decays through non-radiative relaxation with a quantum yield of unity. In this case, $\Delta V_{con} = 0$ and the energy returned to the solvent $Q$, is simply $E_{hv}$ (conservation of energy). The resulting acoustic signal can be described by:

$$S_r = K E_a E_{hv} \left[ (\beta) \left( \frac{1}{\rho C_p} \right) \right] Q$$

(5)
Where $E_{hv}$ is the photon energy of the excitation wavelength. The corresponding ratio of the sample and reference signals then gives:

$$(S_s/S_r)_{E_{hv}} = Q + \left(\rho C_p/\beta\right)\Delta V_{\text{con}}$$

\hspace{1cm} (6)

in which, $Q$ and $\Delta V_{\text{con}}$ can be obtained from a plot of $(S_s/S_r)_{E_{hv}}$ versus $\rho C_p/\beta$. Since, for aqueous solutions $\rho C_p/\beta$ is temperature dependent. The $Q$ and $\Delta V_{\text{con}}$ values can be determined by obtaining acoustic signals for reference and sample as a function of temperature and plotting the amplitude ratios as a function of $C_p\rho/\beta$.

\textbf{Figure 2.1} Overlay of reference and sample acoustic signal with deconvoluted fit to demonstrate frequency shift
The piezoelectric transducer employed in PAC measurements is also sensitive to the frequency of the acoustic signals. For reaction pathways with intermediates between the instrument response time \( \sim 20 \text{ns} \) and the transducer relaxation time \( \sim 15 \mu \text{s} \), for a 2MHz transducer, the frequency of the resulting acoustic signal is shifted from that of the reference (figure 2.1). Since the observed acoustic signals are a convolution of the instrument response function (an underdamped oscillator) \( T(t) \) and exponential heat decay functions, \( H(t) \), described in equation 7 and 8 the magnitude, \( \varphi \), and lifetime, \( \tau_i \), of each event can be determined.\(^2\)

\[
S(t)_{\text{obs}} = H(t) \times T(t) \tag{7}
\]

Where,

\[
H_i(t) = \sum \varphi_i \exp \left(-\frac{t}{\tau_i}\right) \tag{8}
\]

Deconvolution of the signal is accomplished by estimating the parameters \( \varphi \) (equal to \( S/s_0 \)) and \( \tau \) in \( H(t) \) and convoluting the new \( H(t) \) with \( T(t) \), the acoustic signal of the reference. The parameters are varied using a simplex algorithm developed in our lab, until a reasonable chi-squared is reached. Residuals and auto correlation are also \( Q \) and \( \Delta V \) can be obtained from a plot of \( \varphi \) for each event versus \( \rho C_p/\beta \). For the prompt phase \( Q \) is subtracted for \( E_{hv} \) and divided by the quantum yield to get \( \Delta H \) for the process\(^2\)

\[
\Delta H_p = \frac{(Q - E_{hv})}{\Phi} \tag{9}
\]
For every process subsequent

\[ \Delta H_s = -Q \]

Instrument set up, shown in Figure 2.2, involves a Quantum Northwest temperature controlled (to within 0.1°C) 1cm cuvette sample holder mounted with a Panametrics V103 transducer. The detector is coupled to the cuvette by a thin layer of vacuum grease. Photoexcitation of the sample is generated by a 532nm pulse from a Continuum Minilite I frequency doubled/tripled Q-switched Nd:YAG laser (6ns pulse, <
Acoustic signals are amplified with an ultrasonic preamp then recorded by picoscope.

### 2.1.2 Photothermal Beam Deflection (PBD)

The same change in density described above results in a refractive index gradient which can be observed by a deflection in a probe laser beam that can be detected by a position sensor. The change in direction and magnitude of the probe beam deflection is directly proportional to the heat deposited and/or molar volume changes of the sample upon photoexcitation. The total change in refractive index is equal to:\(^2\)

\[ \Delta n = \Delta n_{\text{th}} + \Delta n_{\text{pl}} + \Delta n_{\text{vol}} \]  \hspace{1cm} (11)

Where \( \Delta n_{\text{th}} \) is the contribution due to changes in solvent density from rapid heating, \( \Delta n_{\text{pl}} \) is the contribution due to the ‘population lens’ which is the result of absorption from transient species, and \( \Delta n_{\text{vol}} \) is the contribution due to molar volume and solvation changes of the photoexcited species. The term \( \Delta n_{\text{th}} \) can be examined as long as \( (dn/dT) \) is not dependent on the transient heating temperature:

\[ \Delta n_{\text{th}} = \int (dn/dT) dT \]
\[ \Delta n_{\text{th}} \sim (dn/dT) \Delta T \]  \hspace{1cm} (12)

Where \( \Delta T \) is expressed as equation 1 giving:
\[ \Delta n_{\text{th}} = \frac{\Delta n}{dT} \left( \frac{Q}{\rho C_p} \right) \]  

(13)

Where \( Q \) equaling the thermal energy released to the volume, \( \alpha E_a/hv \)

The second term \( \Delta n_{\text{pl}} \) is described by:

\[ \Delta n_{\text{pl}} = \left( n_0^2 + 2 \right)^2 \alpha / \left( 18n_0 \varepsilon_0 \right) \Delta N \]  

(14)

where \( \varepsilon_0 \) is the vacuum permittivity, \( N \) is the number of excited molecules per unit volume and \( \alpha_i \) is the polarizability of the excited molecules. The population lens arises from absorption of transient species created from photoexcitation. This effects photothermal beam deflection if the sample under investigation or any intermediate species along the reaction pathway exhibit an absorption change at the probe beam wavelength. Therefore it is essential to choose a probe wavelength far from any expected absorption changes of the photoexcited species.

The third term \( \Delta n_{\text{vol}} \) is pertaining to the refractive index change associated with molar volume/solvation changes of the sample can be expressed as:\textsuperscript{2,16}

\[ \Delta n_{\text{vol}} = \int \left( \frac{dn}{dV} \right) dV = \int \left( \frac{dn}{d\varepsilon} \right) \left( \frac{d\varepsilon}{d\rho} \right) \left( \frac{d\rho}{dV} \right) dV \]  

(15)

The term \( (d\varepsilon/dn) = (2n)^{-1} \) from the relationship \( n^2 = \varepsilon \). The \( (dp/dV) \) terms can be found using the ideal gas approximation, \( \rho = N/V \) so that \( (dp/dV) = (d(N/V)/dV) = -N/V^2 = -\rho/V \). The \( (d\varepsilon/dp) \) term is given from the Claussius-Messotti equation:

\[ (d\varepsilon/dp) = (\varepsilon - 1)(\varepsilon + 2)/3\rho \]  

(16)
where $\rho$ is density and $\varepsilon$ is solvent permittivity. Now substituting back into equation 15 gives

$$\Delta n_{\text{vol}} = \int [(\varepsilon-1)(\varepsilon+2)/6 \ \n_n \ ] \ dV \quad (17)$$

where $n_n$ is the refractive index of the solvent. Integrating equation 17 and assuming the changes in volume due to photoexcitation are small ($dV \sim \Delta V$) gives:

$$\Delta n_{\text{vol}} = (\varepsilon-1)(\varepsilon+2)/6 \ n_n (\Delta V/V) \quad (18)$$

Finally substituting equations 13, and 18 into equation 11 gives an expression for the total change in deflection (equation 14 is negligible by choosing probe wavelength far from and anticipated absorbing species):

$$\Delta n = K E_a \Phi \{ (dn/dT) (1/\rho C_p) Q + (\varepsilon-1)(\varepsilon+2)/6 \ n_n (\Delta V/V) \} \quad (19)$$

where $K$ is an instrument response parameter, $E_a$ is the number of Einstein's absorbed, $\Phi$ is the quantum yield for the photochemical process, $Q$ is the heat released to the solvent, and $\Delta V$ is the molar volume change corresponding to any photochemical process. $V(dn/dV)$ equals $(\varepsilon-1)(\varepsilon+2)/6 \ n_n$, simplifying equation 19 gives the corresponding sample deflection:

$$S_s = K E_a \{ (dn/dT)(1/\rho C_p)Q + [V(dn/dV) \ \Delta V] \} \quad (20)$$
Again, the instrument response parameter is unique to each experiment, thus to eliminate $K$ a reference compound is used with the magnitude described by:

$$S_r = KE_a E_{iv} \left[(dn/dT)(1/pC_p)\right]Q \quad (21)$$

Where $E_{iv}$ is the photon energy of the excitation wavelength. The reference compound is chosen so that all of the excitation photon energy is converted into heat and $\Delta V_{con} = 0$. The ratio of the sample and reference signals gives:

$$\left(\frac{S_s}{S_r}\right)E_{iv} = \Phi \left[Q + \Delta V(dn/dV)(pC_p/dn/dT)\right] \quad (22)$$

The $pC_p/dn/dT$ term is temperature dependent allowing the values of $Q$ and $\Delta V$ to be determined from measuring the deflection amplitudes for both the reference and sample as a function of temperature, scaling them to the photon energy and plotting them versus $pC_p/dn/dT$. As well, the $Vdn/dV$ term is constant between 0ºC and 30ºC (the temperature range of the experiments performed here equal to 0.326 (11)). Therefore, $\Delta V$ can be estimated by dividing the slope by 0.326.

For reaction steps occurring after ~10μs, volume and enthalpy changes are obtained by fitting the PBD traces to:

$$F = \alpha_o + \sum \alpha_i (1-\exp(\tau/\tau_i)) \quad (23)$$

where $\alpha_i$ is $S_s$ for each additional process. A plot of $(a/R)E_{iv}$ for each phase versus $pC_p/(dn/dT)$ yields a slope equal to $Q$, where $\Delta H$ for the prompt phase is equal to $Q - E_{iv}/\Phi$ and $-Q$ for the slower phases ($\Phi$ is the quantum yield for each process).\(^2\)
PBD experimental set up is a simple addition to the PAC instrument. The pump pulse is a 532nm pulse from a Continuum Minilite I frequency doubled/tripled Q-switched Nd:YAG laser (6ns pulse, < 80μJ) propagates collinearly with the probe source, an 850nm CW laser. The probe beam is directed using the back mirror to the center of a split photodiode position sensor and the signal is amplified with a home built difference amplifier.

![Graph](image.png)

Figure 2.3 Example of PBD signal fit to equation 23

### 2.1.3 Activation Parameters
A complete thermodynamic profile also requires the knowledge of the activation parameters for each step in the reaction. The activation parameters including ΔG‡, ΔH‡, and ΔS‡ can be obtained by plotting rate constants
associated with each reaction step as a function of temperature using Erying’s equation.²,¹⁷,¹⁸

\[
\ln \left( \frac{k_h}{k_b T} \right) = \frac{\Delta S^\ddagger}{R} - \left( \frac{\Delta H^\ddagger}{RT} \right)
\]  

(24)

Where \( k \) is the observed rate, \( h \) is Plank’s constant, \( k_b \) is Boltzmann’s constant and \( T \) is the temperature. One of the advantages of both PAC and PBD methods is that in order to separate \( Q \) from \( \Delta V \), the measurements must be performed as a function of temperature. Deconvolution of the PAC signals or PBD traces provides the rate constants for the process at each measurement temperature. Thus, the PAC/PBD

![Figure 2.4 Transient absorption spectra at 440nm of CO-myoglobin encapsulated in sol-gel after excitation.](image)
experiments enable the determination of complete thermodynamic profiles for reaction enthalpy. Volume profiles are more problematic as the $\Delta V^\ddagger$ term is found by measuring rate constants as a function of pressure.

### 2.2 Transient Absorption

Transient absorption spectroscopy (TA) probes the change in absorption of a specific chromophore as a function of time after a reaction is initiated by a light pulse. TA is an especially useful tool for examining the complex mechanism of small molecule release and rebinding to heme proteins since the changes in absorption of iron porphyrin in heme proteins can be monitored subsequent to small molecule photodissociation (figure 2.4). However, it is limited to probing dynamics of conformational changes that

![Figure 2.5 Lay out of transient absorption instrumentation](image-url)
effect the heme electronic state and cannot directly measure molar volume and enthalpy changes. Rates obtained from TA measurements can assist in interpreting PAC/PBD measurements by providing information on which phases observed with PAC/PBD involve the heme active site.

Instrument set-up for TA involves a probe source focused through the sample and a pump source intersecting perpendicular to the probe source as shown in figure 2.5. The probe source, white light from a Xe Arc lamp (Oriel), is focused through the sample, into a 1/4m monochromometer, and detected with an Oriel R928 photo-multiplier tube. The signal is then amplified with a wide band-width Melles Griot followed by Stanford Instruments SR445A 350MHz post amplifier and digitized by a Tektronix TDS7404 4GHz digital oscilloscope. The pump source is the second harmonic (532nm) of a Continuum Leopard I Q-switched mode-locked Nd:YAG laser.

2.3 Fluorescence spectroscopy

Fluorescence spectroscopy has proven to be a useful analytical tool in investigating biomolecular dynamics as well as the physical properties of materials. The Perrin-Jabloski diagram illustrated in figure 1.2 in Chapter 1 displays the possible decay pathways of an electronic excited state subsequent to absorption of a photon. In this section the focus is on the emission of a photon from the lowest vibrational level of the $S_1$ electronic excited state called fluorescence. The fluorescence wavelength is always
of less energy and therefore longer than the excitation wavelength due to vibrational relaxation \((h\nu_{\text{ex}} < h\nu_{\text{em}})\) as displayed in figure 2.6. The difference between the two is known as the stokes shift.

\[
S_0 + h\nu_{\text{ex}} \rightarrow S_1
\]

\[
S_1 \rightarrow S_0 + h\nu_{\text{em}} + \text{heat}
\]

Though the absorption and emission of a photon is on the order of \(10^{-15}\)s, excited molecules remain in the S_1 state for picoseconds to a few hundred nanoseconds before decaying through emission of a photon as well as other non-radiative processes. This newly generated population of photoexcited molecules exponentially decays from the S_1 electronic excited state. The time it takes to deactivate the excited-state (the excited state lifetime, \(\tau_s\)) is unique to a given molecule. The fluorescence intensity \(i_f\) at time \(\tau\) after photoexcitation of a short light pulse at \(\tau=0\) is expressed by:

\[
i_f = k_r [A^*]_0 \exp (-\tau/\tau_s), \quad \tau_s = 1/k_r + k_{nr}
\]

(25)

Where \([A^*]_0\) is the concentration of excited molecules at \(\tau=0\), \(k_r\) is the rate constant for radiative deactivation of the S_1 state by emission of a photon, \(k_{nr}\) is the rate constant for all non-radiative deactivation processes. Figure 2.8 displays typical fluorescence lifetime...
traces. The number of photons emitted divided by the number of photons absorbed is defined by the fluorescence quantum yield $\Phi_f$ so that:

$$\Phi_f = \frac{k_r}{k_r + k_{nr}} = k_r \tau_s$$

(26)

which is also unique to given fluorescent molecule.

2.3.1 Time-Resolved Fluorescence Pulsed fluorometry was used to obtain excited state lifetime data. Excitation of the sample is accomplished with a short pulse of light and the subsequent fluorescence decay measured as a function of time. As already mentioned, decay rates are single exponentials, or in more complex situations, the sum of discrete exponentials, or a distribution of exponentials. Time-resolved fluorescence decays were collected on a home built instrument. In this research time resolved fluorescence was used to probe fluorophores adsorbed onto solid state materials and adhered to glass slides which were then placed into a 1cm sample holder oriented 57° relative to the excitation pulse. The excitation pulse was derived from a Continuum Leopard II frequency doubled Nd:YAG laser (< 20 ps pulse width, 532 nm, 20 Hz, ~30 mJ/pulse). The emitted light was passed through a focusing lens followed by a long pass filter and then onto the face of an Electro-Optics Inc., EOT 2030 amplified Si diode (300 ps rise/fall time). The resulting signal was digitized using a Tektronix TDS7404 4 GHz DPO. An example of typical time resolved fluorescence traces are displayed in figure 2.8.

2.3.2 Steady-State Fluorescence Intensity The steady-state population of excited molecules is due to the fact that the pseudo-first order rate constant for light absorption
is $k_a \approx 10^{15} \text{ s}^{-1}$ and de-activation is on the order of $10^7-10^{10} \text{s}^{-1}$. The measured steady-state fluorescence intensity per absorbed photon can be expressed as a function of wavelength of the emitted photons, $F_\lambda(\lambda_t)$ keeping the relationship:

$$\int F_\lambda(\lambda_t) \, d\lambda_t = \Phi_F, \text{ integrating over } 0<\lambda_t<\infty$$  \hspace{1cm} (27)$$

Where $F_\lambda(\lambda_t)$ is the fluorescence spectrum. The fluorescence spectrum represents the probability of the transitions from the lowest vibrational $S_1$ excited state to various vibrational levels of the ground state. Every spectrofluorometer has a different optical configuration, i.e, the solid angle the instrument collects fluorescence, the bandwidth on the monochromator, high voltage on the photomultiplier tube, etc. Therefore, in practice the steady-state fluorescence intensity $I_F(\lambda_F)$ is described by:

$$I_F(\lambda_E, \lambda_F) = kF_\lambda(\lambda_F)I_\lambda(\lambda_E)$$ \hspace{1cm} (28)$$

Where $k$ is the proportionality constant related to the optical configuration, $I_F(\lambda_F)$ is measured at wavelength $\lambda_F$ and $I_\lambda(\lambda_E)$ is the difference between the the intensity of the incident light $I_0(\lambda_E)$ and the intensity of the transmitted light $I_T(\lambda_E)$. The intensity is can also be written in terms of absorption of the incident beam intensity:

$$I_F(\lambda_E, \lambda_F) = kF_\lambda(\lambda_F)I_0(\lambda_E)\{1-10^{-A(\lambda_E)}\}$$ \hspace{1cm} (29)$$
Where $\lambda_E$ is the excitation wavelength, $A(\lambda_E)$ is the absorbance at the excitation wavelength.

An ISS PC1 spectrofluorimeter was used in collecting steady-state fluorescence data.

2.3.3 Fluorescence Polarization and Anisotropy

Fluorescence polarization and anisotropy measurements are utilized to gain information regarding size, shape, rotational mobility, etc. of emitting molecules. Fluorescence polarization and anisotropy utilize the fact that chromophores absorb light parallel to the absorption transition moment of a particular electronic state and emit according to the emission transition moment. The emission transition moment is independent of the excited states reached upon excitation ($S_n$) due to internal conversion to the lowest vibrational excited state $S_1$. Therefore if the incident light is linearly polarized then only the population of molecules whose absorption transition moment is of the same direction of the electric field are preferentially excited. The emission is then also polarized however, if the direction of the fluorophore changes within the excited state lifetime then the fluorescence is said to be depolarized.

In practice the polarization is collected by exciting with light vertically polarized and observing the fluorescence at right angles to the horizontal plane\textsuperscript{19,20}:

\[
P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}
\]  

(30)

And anisotropy is expressed as:
\[ r = I_\parallel - I_{\perp} / (I_\parallel + 2I_{\perp}) \]  \hspace{1cm} (31)

Polarization measurements were performed on a ISS PC1 spectrofluorometer.

2.4 References


Chapter 3: System I-Horse Heart Myoglobin Encapsulated Sol-gels

3.1 Background.

In order to achieve a complete physiological understanding of heme protein function it is necessary to design experiments that mimic physiological conditions. Cellular environments within which heme proteins function have minimal volume available due to immense crowding from an array of additional biomolecules essential for overall cellular function. Unfortunately, even with knowledge of physiological conditions and their critical significance on protein function, heme proteins are often probed in dilute solution conditions and not under restricted volume conditions.\(^1-^5\) Although solution experiments have provided a plethora of information regarding the mechanism through which heme proteins function, recently there has been an increase toward probing heme protein systems in confined space. Encapsulating biomolecules (peptides, proteins, enzymes, etc) in environments where bulk solvent and space are controlled can be used to identify the importance of these properties in the conformational dynamics of their physiological function.

While encapsulation of biomolecules allows for a more physiologically relevant platform to examine heme proteins, of equal interest is the use for the development of novel biosensors and biocatalysts. Today biocomposite materials are employed in many commercial processes including the manufacture of glycerides, phospholipids, peptides, amino acids, antibiotics, etc. Biosensors and biodiagnostics have also been developed...
to detect sugars, ascorbate, amino acids, antigens, ammonia, nitrogen monoxide pesticides and pathogens to name a few. Nonetheless, bioimmobilization has proved important for many applications. The technology of utilizing inherent properties of biomolecules to perform particular chemistries and/or bind specific analytes to use as sensors comes with the need to easily separate them from bioproducts, and retain high activities and long term stability. Currently, an array of biomolecules including, myoglobin, hemoglobin, horseradish peroxidase, glucose oxidase, etc have been encapsulated in an enormous library of solid state platforms including layered clays, Nafion™, sugar glasses, etc, however of particular interest for this work are sol-gels.

\[
\begin{align*}
\text{Acid or base} & \quad \text{Hydrolysis} \\
& \quad 1. \ (\text{RO})_3\text{Si-CH}_3 + \text{H}_2\text{O} \rightarrow \ (\text{RO})_3\text{Si-OH} + \text{CH}_3\text{OH} \\
& \quad \text{Water Condensation} \quad 2. \ (\text{RO})_2\text{Si-OH} + (\text{RO})_2\text{Si-OH} \rightarrow (\text{RO})_3\text{Si-O-Si(RO)}_3 + \text{H}_2\text{O} \\
& \quad \text{Alcohol condensation} \quad 3. \ (\text{RO})_3\text{Si-CH}_3 + (\text{RO})_3\text{Si-OH} \rightarrow (\text{RO})_3\text{Si-O-Si(RO)}_3 + \text{CH}_3\text{OH}
\end{align*}
\]

**Figure 3.1** Scheme of the sol-gel processes, where R is CH₃ for tetramethyl orthosilicate

Sol-gels are silica based optically clear porous materials. The sol-gel process involves hydrolysis, condensation and polycondensation of a liquid alkoxide precursor, as shown in figure 3.1. The research presented here involves sol-gels derived from the hydrolysis of tetramethylorthosilicate in the presence of water under acidic conditions to form hydrated silica tetrahedra. Condensation and further polycondensation forms a porous silicate matrix, capable of trapping biomolecules, with interconnecting channels to the bulk solvent allowing for diffusion of small molecules throughout the gel. Of critical importance to biomolecule encapsulation is the fact that sol-gel polymerization occurs around incorporated species (templating effect) ensuring one molecule per pore and the average size and water content of the pores can be controlled by the amount of
water present during the hydrolysis process. Viscosity measurements performed by Narang et al. revealed a significant increase in viscosity with aging time. Interestingly, early aging times (similar to the ones probed herein) exhibit a microviscosity of about 2cP. Neat water at 20ºC has a viscosity of 1.0020cP. Another characteristic of sol-gels is at pH 7 the SiOH groups lining the walls are deprotonated and negatively charged which likely order the few solvation layers also present. In general the sol-gel process employed in this research produces pH 6.5 sol-gels with an average pore with a diameter of 100Å. It should also be noted that with all the sol-gel process available and variations in conditions during synthesis as well as the aging time data is collected, caution must be taken when comparing results from one investigation to the next. Still, they are ideal for immobilization of biological molecules, since polymerization occurs around the protein creating a pore within which they are protected from aggregation, unfolding, and diffusion. The hydrophilic nature of these silica matrices produces favorable environments for biomolecules, and ensures small molecule diffusion into the

![Figure 3.2 Schematic drawing of Mb encapsulated in sol-gel. Not to scale](image)
populated cavities. Furthermore, sol-gels are chemically inert, and possess both thermal and mechanical stability which is crucial when probing stress on protein function. In some cases, sol-gel encapsulated proteins displayed increased pH and thermal stability.27,28,33,34

To date an array of biological molecules have been encapsulated in sol-gels, including cytochrome C, glucose oxidase, myoglobin, creatine kinase, etc.35,36 Most of the trapped biomolecules retain equal or better activity than their free counterparts, as well as, display enhanced stability towards temperature, solvent, and pH. For example, sol-gel encapsulated creatine kinase displayed 50% of its activity for ten times longer than its free enzyme, in addition to increased thermal stability.3,4 Alkane phosphatase retained activity as low as pH 0.9, when it optimally performs at pH 9.5. Caged biomolecules are typically surrounded by only a few solvent layers, thus at low pH the number of protonated waters, H$_3$O$^+$, present is not enough to cause significant perturbations (unfolding) to the enzyme which improves pH stability.3,4

Horse heart myoglobin (Mb) has been extensively utilized as a model heme protein system to better understand the structure-function relationship of small molecule binding.37-47 Frauendenfelder has even named myoglobin the Hydrogen atom of biology.48 The protein structure was the first available at high enough resolution to give a complete set of atomic coordinates unveiling the three-over-three α-helical globin fold. Myoglobin is an oxygen storage heme protein consisting of an iron protoporphrin IX active site coordinated to the protein through a proximal histidine residue located deep within a hydrophobic pocket (figure 3.3). To date, many experimental investigations as well as extensive computational studies have provided a strong foundation for protein dynamics of ligand migration in myoglobin.7,8 Still a clear understanding of precisely how
O₂ migrates from the heme iron to the solvent, and likewise from the solvent to the heme iron does not exist.

Upon excitation the heme iron back donates electron density to anti-bonding orbitals of gaseous ligands thereby initiating ligand migration. Carbon monoxide, though non-physiological for myoglobin, is often experimentally preferred over other gaseous ligands including O₂ or NO. The photodissociation of CO has an electronic barrier causing low geminate rebinding and thus high quantum yield (near unity) of photocleavage from the ferrous heme. Experimental techniques including optical and vibrational spectroscopies, electrochemical methods, differential scanning calorimetry, etc have provided key insights on the conformational dynamics of CO migration under dilute conditions or solution conditions. The overall accepted mechanism for physiological temperatures developed from these experiments as well as X-ray crystallography begins with CO bound and the protein structure in a closed conformation with His64 rotated in the protein pocket forming a hydrogen bond network with H₂O -
Lys45 - 6-propionate group of the heme (no obvious channel for ligand exit). Within picoseconds of CO photorelease the ligand migrates to a nearby distal site. The ligand remains near the distal side of the heme for 100-300ns until it traverses to Xe(1) binding pocket. There is still some disagreement with the final escape to the solvent, however at physiological temperatures it appears that CO exits through a His ‘gated’ channel with a lifetime of ~800ns for hhMb. The His64 -H₂O - Lys45 - 6-propionate group of the heme h-bond network is disrupted by rotation of His 64 out of the distal pocket to form a migration pathway open to the bulk solvent. In addition X-ray crystallographic data displays a water molecule bound to His64 (~84% occupancy) indicating the entry of a water molecule following CO exit. This water molecule is believed to govern CO entry to the protein matrix. A sequential two barrier model has previously been proposed for CO rebinding kinetics. The outer barrier is characterized by CO entry and the inner barrier assigned to ligand binding to the heme. Specific heat measurements by Kleinert and Authors concluded that the outer barrier is dependent of viscosity while the inner kinetic barrier is not. In addition, it is believed that the inner barrier ultimately controls rebinding kinetics to the heme active site.

The general use of CO for elucidating ligand migration in myoglobin is also employed in numerous theoretical studies. Recently a theoretical study by Ruscio et al examined CO migration exit and entry to the protein matrix at room temperature. The MD simulations revealed two distinct dynamic pathways for CO migration to the solvent. Multiple trajectory simulations displayed that CO migrates more frequently down one path (major) than the other (minor). Trajectory simulations of CO re-entry also displayed preference down the major pathway. For both exit and entry ligand migration down the major pathway was accompanied with short lived ligand docking involving the Xe(4) and Xe(1) cavities of myoglobin. These results are in good agreement with previous time-
resolved experiments, including time-resolved X-ray studies of CO photodissociation from ferrous myoglobin. Ruscio et al also revealed multiple residues involved in exit portals for CO, however the His portal was dominate over the rest. While these results are consistent with experimental results, according to this work other exit portals to the solvent are possible.

Previous photothermal methods have shown that flash photolysis of Mb-CO in pH8 buffered solution conditions results in formation of two fast intermediates (τ<50ns, and τ=80ns). Angeloni and Feis reported, within the prompt phase (<50ns), photocleavage of CO forms a fast intermediate deoxymyoglobin with CO docked near proximal heme pocket, resulting in a volume contraction of -3mL mol⁻¹ and a positive enthalpy change of ~14 kcal mol⁻¹ nearly that of the enthalpy change for CO-Fe bond dissociation (17kcal mol⁻¹). This was followed by the 80ns fast relaxation measured to have a ΔV= -3ml mol⁻¹ and a ΔH= -3kcal mol⁻¹. The authors assigned this phase to CO migration from the primary docking site or a Xe docking site to another Xe docking site. Migration of the ligand out of the distal pocket reduces repulsive interactions between the CO oxygen and the Nε hydrogen of His64 significantly contributing to the overall observed relaxation. Subsequent to the prompt 80 ns phase, Angeloni and Feis observed an ~800ns phase with ΔH_{struct} ~11kcal mol⁻¹ and ΔV s=3±1 ml mol⁻¹ assigned to the protein motions, including breakage of a salt bridge between Lys45 and the heme 6-propionate group, open an access channel for CO to escape to the surrounding solvent and a subsequent water molecule to enter the protein matrix.41

More recently, the encapsulation of myoglobin in solid state matrices (nafion, sol-gels, zirconium phosphate,etc.) has allowed for a more physiologically relevant study of proteins in confined space. In addition, it has been shown that some solid state matrices have the ability to trap metastable species that are otherwise too fast to
observe with traditional solution studies. Previous studies by Sottini and authors of CO rebinding kinetics to myoglobin doped silica gels in the presence of glycerol revealed an increase in geminate rebinding when compared to solution conditions\textsuperscript{33}. Furthermore, they found a strong dependence of geminate yield on glycerol concentration and temperature, suggesting that molecular confinement of the gel matrix reduces protein fluctuations necessary for CO solvation. Another investigation by Samuni and authors investigated equilibrium and non-equilibrium conformational populations of myoglobin using sol-gels and varying concentrations of glycerol.\textsuperscript{34} Two encapsulation protocols were examined utilizing the resonance Raman frequency of $\nu$(Fe-His) of the iron-proximal histidine stretching mode. Spectroscopic and kinetic differences were observed between sol-gel encapsulated deoxyMb with CO bound after encapsulation and directly encapsulated COMb. These results indicate the ability to lock specific myoglobin conformations. The authors stated that the ability to lock myoglobin conformations is not only an effect of viscosity, but also attributable to limitation of space and stability of the protein solvent shell due to interactions with the Si-O$^-$ groups bordering the cavities.

Though thermodynamic profiles are necessary to have a complete understanding of complex ligand binding mechanisms, profiles of ligand binding/release to myoglobin confined in solid state materials have not yet been accomplished. In fact to our knowledge this is the first attempt to investigate the effects of confinement on a biological system encapsulated in sol-gels using PAC and PBD. The vast knowledge of ligand binding to Mb in solution as well as the extensive catalog of information on sol-gels make this an ideal system. With the use of photoacoustic calorimetry we report molar volume changes and kinetics associated with physiological process involved in CO dissociation from horse heart myoglobin encapsulated in sol-gels.
3.2 Sample Preparation for PAC

All materials were purchased from sigma Aldrich and used without further purification. A description of PAC and PBD can be found in Chapter 1- Methods.

To prepare sol-gels for PAC measurements, 1 volume 2mM HCl is mixed with 2 volumes tetramethyl orthosilicate (TMOS) in a glass vial. The mixture is vortexed then placed in the refrigerator for 1 hour. To form Mb sol-gels, 1 ml of 50mM phosphate buffer, pH 6.5, containing Met-Mb is added quickly to 1 ml of the cool TMOS solution in a plastic cuvette and a metal wire is inserted as shown in figure 3.1 (final OD$_{532\text{nm}}$ ~0.5). Once the sol-gel begins to harden a layer of buffer is added to the top to slow the gelation process. For PAC measurements the hardened gel is sealed with a septum cap and deaerated for 20 min, with an argon purge, upon which time the sample is then reduced with sodium dithionite to form the Fe(II) (deoxy) Mb. The deoxy sample is quickly transferred to the quartz cuvette in the PAC sample holder, layered with deareated buffer, sealed with a home built cap, and a small amount of sodium dithionite is added to remove any residual oxygen from transfer. The deoxy sample is not photo-active and degrades absorbed photon energy with unity quantum yield thus serving as a calorimetric reference. Once PAC data is collected at

![Figure 3.4 PAC set-up for block Mbsol-gels. Detector and cuvette are couple with a thin layer of vacuum grease](image)

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various temperatures, the deoxy sample is purged with CO using a homebuilt cap without perturbing the detector interface. After 1 hr of incubation with CO the deoxy Mb is converted to COMB and PAC measurements are obtained at the same temperatures as the reference.

As already discussed in Chapter 2 section 2.1.1, to obtain $Q$ and $\Delta V_{con} C_p \rho / \beta$ for the solvent must be known. Unfortunately they are not known for for 50 mM phosphate, pH 6.5, sol-gels from 18°C - 34°C sol-gels and must be calculated. Since the exact value for aqueous solutions have been determine for the temperature range we are examining the acoustic signal for a calorimetric reference can be directly compared to a calorimetric reference encapsulated in a sol-gel using the relationship:

$$(C_p \rho / \beta)_{ref} R_{ref} = (C_p \rho / \beta)_{solgel} R_{solgel}$$

(1)

where $R$ is the measure acoustic amplitude. The solvent parameters are different for every sol-gel therefore this calculation must be performed for every experiment. This is accomplished by obtaining the acoustic signals for the reference sol-gels (deoxy) and a second reference compound in deionized water under identical PAC instrumental conditions

3.3 PAC Results:

Figure 3.2, top panel, displays an overlay the acoustic signal for CO flash photolysis from Mbsol-gel at 25°C and the fit obtained from deconvolution using software developed in our lab and residuals. The auto correlation in included in the inlay to illustrate goodness of fit. The sample signal is shifted in frequency relative to the reference signal indicates multiple phases upon CO photodissociation. Deconvolution of the signals provides lifetimes for two phases, a prompt phase $\tau_p < 50$ns and a slower
phase with a τ~960ns. In order to obtain volume and enthalpy from plots of the amplitudes (φ₁ and φ₂) of the two phases as a function C_pρ/β. To determine reaction volume and heat released for each phase the temperature dependence of the φs is fit to equation 6 from chapter 2 section 2.1.1 (figure 3.5 bottom panel). The prompt phase is calculated to have a volume change of 4±1 mL mol⁻¹ with an enthalpy change of 19±5 kcal mol⁻¹ for a quantum yield of one. The slower phase is exothermic with an enthalpy change of -6±6 kcal mol⁻¹ associated with a change in volume of 4±1 mL mol⁻¹. The temperature dependence of the rate constants for the slow event can be used to derive activation enthalpy and entropy as described in equation 24 in chapter 2 section 2.1.3. The calculated activation parameters are ΔH‡ = 13±2. kcal mol⁻¹ and ΔS‡ = 12±3.5 cal mol⁻¹.

3.3.1 Discussion. In this study, photoacoustic calorimetry was employed to investigate the enthalpy and volume changes associated with CO release from hhMb encapsulated in sol-gel. Steady state absorption spectra of Mb³⁺, Mb²⁺, and Mb²⁺CO encapsulated in sol-gels are indistinguishable from solution spectra, indicating the process of encapsulation does not perturb the heme environment. Therefore, as with Mb
in neutral solution, the heme iron is 6-coordinate with His93 bound on the proximal side and CO bound in the sixth position. Still, in dissecting the enthalpy and volume changes associated with CO photorelease from hhMbsol-gel it becomes evident that the results differ from those in solution at slightly basic pH. However, the steady-state optical spectra only probe equilibrium states. As will be discussed below the time resolved thermodynamics indicate perturbations to the dynamics associated with ligand release.

### Table 3.1 Summary of PAC results for CO photodissociation from Mb in solution and sol-gels.
The prompt phase for solution, pH 8 is the sum of the prompt and fast phases observed by Angelioni and Feis.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$\Delta V_p$ ($\text{ml mol}^{-1}$)</th>
<th>$\Delta H_p$ ($\text{kcal mol}^{-1}$)</th>
<th>$\Delta V_s$ ($\text{ml mol}^{-1}$)</th>
<th>$\Delta H_s$ ($\text{kcal mol}^{-1}$)</th>
<th>$\tau_s$ (ns, $20^\circ C$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution, pH 8</td>
<td>-6.0±0.2</td>
<td>11±1</td>
<td>19±1</td>
<td>6±7</td>
<td>800</td>
</tr>
<tr>
<td>Solution, pH 3.5</td>
<td>2.2±0.1</td>
<td>14±1</td>
<td>2.1±0.1</td>
<td>3.3±0.2</td>
<td>230</td>
</tr>
<tr>
<td>Sol-gel</td>
<td>3.2±1.5</td>
<td>18±2</td>
<td>3±1</td>
<td>-5±4</td>
<td>960</td>
</tr>
</tbody>
</table>

### 3.3.1.1 Prompt phase.

As mentioned in section 5.1, previous photothermal studies have shown that CO photorelease from Mb-CO in pH8 results in formation of two fast intermediates ($\tau<50\text{ns}$, and $\tau=80\text{ns}$). Under these conditions CO-hhMb is in a closed conformation with no obvious ligand migration path to the bulk solvent, accordingly protein fluctuations are required for ligand exit. Angeloni and Feis reported, within the prompt phase ($<50\text{ns}$),
photocleavage of CO forms a fast intermediate deoxymyoglobin with CO docked near the proximal heme pocket, resulting in a volume contraction of -3mL mol$^{-1}$ and a positive enthalpy change of $\sim$14 kcal mol$^{-1}$ which is nearly that of the enthalpy change for CO-Fe bond dissociation (17 kcal mol$^{-1}$). The prompt phase is followed by the 80ns fast relaxation with $\Delta V = -3$ ml mol$^{-1}$ and $\Delta H = -3$ kcal mol$^{-1}$. The authors assigned this phase to CO migration from the primary docking site or a Xe docking site to another Xe docking site. Migration of the ligand out of the distal pocket reduces repulsive interactions between the CO oxygen and the $N_e$ hydrogen of His64 significantly contributing to the overall observed relaxation. In contrast, sol-gels made using 50mM phosphate buffer, pH 6.5, exhibit only one fast phase occurring faster than the time resolution of the instrument (i.e. <50ns) with an integrated $\Delta V_p = 3 \pm 1$ ml mol$^{-1}$ and $\Delta H_p = 18 \pm 2$ kcal mol$^{-1}$. The lack of an 80ns phase in Mbsol-gels, could result from Mb interactions that cause this event to occur on a faster time scale, i.e. within the time window. In this case, the sum of the prompt phase and 80ns phase $\Delta H$ and $\Delta V$ values would match those obtained for the sol-gel material. As evident in table 3.1 the $\Delta H$ and $\Delta V$ values are quiet distinct for the Mbsol-gel material. In contrast, it has also been shown that under acidic solution conditions, myoglobin adopts a more open conformation with disruption of the lys45-asp60 salt bridge and protonation of His64. The previous PAC studies by Angeloni and Feis of Mb at pH3.5 establish that the open conformation does not display the 80ns fast phase. In addition CO release to the bulk solvent occurs with a much faster rate $\tau \sim 230$ns. The authors report an enthalpy and volume change associated with the prompt event of $\Delta H_p = 13.9 \pm 1.4$ kcal mol$^{-1}$ and $\Delta V_p = 2.2 \pm 0.1$ mL mol$^{-1}$. Which are consistent with Mbsol-gel data. The fast that the Mbsol-gel results for the prompt phase are similar to those obtained for an 'open' conformation at low pH would suggest that either the solgel
cavities are mildly acidic or that the solvent-protein-sol-gel interactions promote an open conformation.

In order to establish the pH associated with the interior cavaties, a pH dependence study was conducted on dichlorofluorescein doped sol-gels (DCFsol-gel). Dichlorofluorescein is a common fluorescent pH indicator used to probe the pH range under question (pK_a~4). The steady-state emission spectrum was obtained for a DCFsol-gel prepared and aged the same as with the PAC Mbsol-gel measurements to ensure the microenvironments of the cavities were similar. The results indicate the sol-gel procedure employed here produces slightly acidic cavities of pH ~6.5, identical to that of the buffer used during synthesis. Furthermore, the heme proximal His93 bond breaks, becomes protonated and consequentially replaced by a water molecule below pH 4 in metMb. If this were evident here, a change in the ligation state of the heme iron would be seen in the steady state absorption spectra of the metMb sol-gel. These results are further supported by a resonance Raman study by Kanti Das and authors, which found that the \( \nu_{Fe-His} \) mode for metMb encapsulated in sol-gels synthesized with pH 7 buffer is identical to neutral solution studies. Therefore, the observed thermodynamics of the prompt phase are consistent with sol-gel encapsulation perturbing the protein tertiary structure giving rise to conformation dynamics similar to those observed for Mb in acidic solutions and not due to actual decrease in pH during synthesis. The SiOH groups lining the interior walls of the silica gels are deprotonated and negatively charged at neutral pH making the possibility for increased solvent order and electrostatic interactions with the protein surface.
3.3.1.2. Slow Phase

The PAC measurements of CO photo-released from Mbsol-gels also reveal a slow process with a lifetime of 960 ns at 20ºC, which is assigned to ligand migration out of the protein matrix. This kinetic event gives rise to a change in enthalpy of -5±4 kcal mol⁻¹ and a small positive volume change of 3±1 mL mol⁻¹. Crystallographic data of deoxy mb suggests that CO escape to the bulk solvent is followed by the entry of a water molecule into the heme pocket, due to the fact that a water molecule is hydrogen bonded to the nearby His64 with 80% occupancy in the deoxy state and not in the CO bound Mb.⁹,¹⁹ It is believed that the water entry is governed by CO escape out of the protein interior which requires disruption of the Lys45-Asp60 salt bridge resulting in a change from the closed form to the open conformation and allowing for direct access between the heme distal pocket and the bulk solvent. The observed enthalpy, ΔΗₘ, originates from: 1) CO escape to the bulk solvent and subsequent solvation, 2.) H₂O entry to the distal heme pocket H-bonding with the nearby His63 and 3.) protein conformational dynamics thus, ΔΗₘ = ΔΗ₇₃₂₄_out + 0.8ΔΗ₂ₒ₂_in + ΔΗ_struct, where ΔΗ_struct includes any protein conformational fluctuations, bond breakage, solvent reorganization, etc., ΔΗ₇₃₂₄_out corresponds to CO solvation (-2.6 kcal mol⁻¹) and ΔΗ₂₀_in is the enthalpy change associated with the water entry and hydrogen bonding to His64 (-7 kcal mol⁻¹).²⁰ The value of ΔΗ_struct (=ΔΗₘ - ΔΗ₇₃₂₄_out - 0.8ΔΗ₂ₒ₂_in ) is indicating minimal conformational change upon CO release to the bulk. In comparison, solution studies at pH 3.5 and 8 result in endothermic ΔΗ_struct of ~8 kcal mol⁻¹ and ~11 kcal mol⁻¹, respectively. These results indicate that the local environment in the sol-gel cages are not conducive for major solvent reorganization necessary for large protein fluctuations following CO photolysis, but still allow CO escape from the protein pocket.

The effects of encapsulation are also evident in the observed change in volume and lifetime for the slow process. The experimental ΔVₘ for hhMbsol-gel, solution pH 8,
and pH 3.5 are 3±1 ml mol⁻¹, 19±1 ml mol⁻¹, and 2.1±0.1 ml mol⁻¹, respectively with the lifetime for each of 960ns, 800ns and 250ns at 20°C. Similar to the ΔHₘₐₓ analysis, the ΔVₘᵢₙ values can also be estimated using ΔVₘᵢₙ = ΔVₘᵢₙ - ΔVₐₐₜₜₜ - 0.8ΔVₐₐₜₜₜ, where Vₐₐₜₜ = 35 mL mol⁻¹ (59) and Vₐₚₚₚ = 18 mL mol⁻¹. Thus the change in volume associated with conformational dynamics upon CO photorelease are -18 ml mol⁻¹, -22 mL mol⁻¹, and -6 mL mol⁻¹ for Mbsol-gel, solution pH3.5 and solution pH8 respectively. The volume contraction observed in sol-gel encapsulation is much more than solution studies at pH 8 but is comparable again to pH 3.5. However, the lifetimes of the slow phase further supports that the dissimilarities between solution pH 8 and sol-gel made with pH 6.5 buffer are not due to acidic confines but more likely protein-solvent-sol-gel interactions.

### 3.4 PBD Results

Figure 3.7 displays a PBD trace for CO rebinding to sol-gel encapsulated deoxyMb at 20°C. The initial rise in the amplitude, occurring less than 10μs, is accompanied by a slower millisecond phase associated with CO rebinding. The traces for each temperature from 15-35°C were fit to equation 23 in Chapter 2 Section 2.1.2 to obtain amplitudes for the fast and slow phases. The temperature dependence of the amplitudes for the two phases are plotted in figure 3.8 top panel and the corresponding volume and enthalpy changes are summarized in table 3.2. The experiments were repeated under identical solution conditions and the results are shown in figure 3.7 bottom panel and included in table 1.
3.4.1 Discussion: The method of PBD is complimentary to PAC in that it probes the time-resolved thermodynamics of processes on longer time scales (~10μs to ms). Therefore, PBD can be used to study the enthalpy and volume changes as well as time scales of conformational dynamics associated with CO rebinding to Mbsol-gel. A direct comparison between the results for PAC and PBD on COMBsol-gels should not be done since they involve two different sol-gels and it should not be assumed the Mb environments for both are identical.

3.4.1.1 Prompt Phase

The prompt phase of PBD consists of the phases that occur after CO dissociation from the heme to ~10μs. In short, this phase is the sum of the events, prompt and slow phases, described above in the PAC section. During this time CO is photoreleased, migrates away from the active site, eventually escapes the protein matrix, solvates and is followed by immediate water entry. The PBD results show that the prompt phase is an endothermic reaction for both Mb in solution and sol-gel encapsulated under the same conditions, 50mM phosphate buffer pH 6.5 (ΔH=11±1 kcal mol⁻¹ and 19±2 kcal mol⁻¹, respectively). CO–Fe bond cleavage has an estimated ΔH equal to 17kcal mol⁻¹, indicating that the
prompt phase enthalpy in the sol-gel is dominated by this contribution. However, CO solvation also requires opening of the protein pocket by rotation of His64 and disruption of a salt bridge. Transition to the open form Mb exposes charged species to the solvent, consequently, the solvent response (i.e., electrostriction) may play a role in the differences observed between sol-gel and solution due to the inconsistencies in solvent conditions (ionic strength in the cages versus solution) and/or encapsulation may also induce a more open form reducing the global changes necessary for CO release. A more open conformation is predicted by the PAC data.

Table 3.2 Summary of PBD data

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$\Delta V_p$ (mL mol$^{-1}$)</th>
<th>$\Delta H_p$ (kcal mol$^{-1}$)</th>
<th>$\Delta V_s$ (ml mol$^{-1}$)</th>
<th>$\Delta H_s$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution</td>
<td>6.3±0.1</td>
<td>11±1</td>
<td>-6.3±0.9</td>
<td>-11±1</td>
</tr>
<tr>
<td>Sol-gel</td>
<td>1.2±0.5</td>
<td>19±2</td>
<td>-1.5±0.5</td>
<td>-18±1</td>
</tr>
</tbody>
</table>

The corresponding changes in volume related to these events are large and positive for Mb in solution (6.3±0.8 ml mol$^{-1}$) and small for sol-gel encapsulated Mb (1.2±0.5 ml mol$^{-1}$). Though the magnitudes of the thermodynamic parameters discovered with PAC cannot be compared directly, the trends are in agreement with PBD in that structural volume changes are on the same order as COMb pH 3.5 solution studies suggesting that the lack of physical space is not entirely to blame on the differences between sol-gel and solution. However, the possibility of solvent reorganization not permitted in the confines of the sol-gel pores cannot be ignored. The time resolution of PBD does not allow us to see the intermediates involved with CO dissociation, only the overall changes in enthalpy and molar volumes.
3.4.1.2. Slow Phase

The slow phase observed by PBD displays an enthalpy and volume change of $-18\pm1\text{kcal mol}^{-1}$ and $-1.5\pm0.5\text{ mL mol}^{-1}$, respectively, with a lifetime of $\sim2\text{ms}$ at $25^\circ\text{C}$. The fact that these results are equal and opposite to those associated with CO dissociation (the prompt phase) tells us the process is reversible and must involve CO rebinding. Previously, the enthalpy of CO bond formation to chelated hemes was reported as $-17\text{kcal mol}^{-1}$ indicating the global changes of the system upon CO rebinding are minimal.

Both PBD and TA data display the rebinding of CO as one conformational event with a rate constant of $5.7\times10^2\text{ s}^{-1}$ at $25^\circ\text{C}$, and second order rate constant of $5.7\times10^5\text{ M}^{-1}\text{s}^{-1}$ under 1atm of CO. The temperature dependence of the rate constants obtained from equation 9 in chapter II can be used to determine the activation parameters for CO rebinding using the equation:

$$\ln \left( \frac{k_h}{k_bT} \right) = \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT}$$  \hspace{1cm} (5)

Preliminary activation enthalpy and entropy obtained are equal to $4\pm2\text{ kcal mol}^{-1}$ and $-15.9\pm3.8\text{ cal mol}^{-1}\text{ K}^{-1}$, respectively. The activation parameters are similar to previous PBD solution studies, $\Delta H^\ddagger=7.1\pm0.8\text{ kcal mol}^{-1}$, and $\Delta S^\ddagger=-22\pm3\text{ cal mol}^{-1}\text{ K}^{-1}$. The similarities in these results between the solution studies and sol-gel suggest that lack of bulk solvent, space and possible interactions with the sol-gel SiO$^-$ groups do not significantly affect CO re-entry and migration through the protein matrix .

Data obtained from PBD provides information on global changes and is not limited to changes associated with the heme chromophore. The PBD traces are monoexponential and display similar rate constants and activation parameters as with CO rebinding detected with optical spectroscopic techniques. Therefore, the
thermodynamics of the single event observed by PBD correlates to Fe-CO bond formation and that ligand migration from entry point to binding site do not induce major changes in enthalpy and volume.

### 3.5 Effects of Confinement

To summarize the above results, upon CO photodissociation from Mbsol-gel PAC observes a prompt phase with an estimated volume expansion of $3\pm1 \text{mL mol}^{-1}$ and endothermic enthalpy change of $19\pm5 \text{kcal mol}^{-1}$. During this time, <50ns, the Fe-CO bond is cleaved and CO has migrated away from the active sight but not exited the protein matrix. An exothermic slow phase ($\tau_s\approx960 \text{ ns}$) follows with an enthalpy change of $-5\pm4 \text{kcal mol}^{-1}$ associated with an expansion of $3\pm1 \text{ mL mol}^{-1}$, which is assigned to CO escape from the protein matrix. These results would indicate that the encapsulated protein adopts a more open conformation. The lifetime of the slow phase $\tau\approx960 \text{ ns}$ would indicate a different open conformation than at pH3.5. PBD also observes these phases integrated in the prompt phase, revealing a $\Delta H=19\pm2 \text{kcal mol}^{-1}$ and $\Delta V=1.2\pm0.5 \text{ ml mol}^{-1}$. The reversible process of CO rebinding exhibits a $\Delta H=-18\pm1 \text{kcal mol}^{-1}$ and $\Delta V=-1.5\pm0.5 \text{ ml mol}^{-1}$ with a $\tau\approx1.5 \mu\text{s}$. The results for both methods were compared to solution studies and the differences are highlighted in figure 3.9.
Figure 3.9  Thermodynamic profiles for CO rebinding to Mbsol-gel, Mb pH8 solution, and Mb solution pH3.5 obtained from PAC. Thermodynamic profiles for CO rebinding to Mbsol-gel, Mb pH8 solution, and Mb solution pH3.5 obtained from PBD. Molar volume changes are shown in blue and enthalpy changes are shown in red. Molar volume changes are shown in blue and enthalpy changes are shown in red.
Though it is not obvious how sol-gel encapsulation perturbs the conformational dynamics of CO photorelease from Mb, the fast (PAC) and slow phase (PBD) results are consistent with three possible effects of confinement: 1.) increased microviscosity in the pores 2.) electrostatic interactions with the protein surface and the SiO$_2$ groups, and 3.) solvent shell stability due to the negatively charged borders of the pores. A highly viscous environment is expected based on the estimated pore diameter of ~100Å as compared to 45Å for myoglobin. Previous solution studies found that the rates of CO escape and CO entry exhibit viscosity dependence. As well the open conformation Mb under pH 3 solution conditions exhibits a CO escape lifetime of $\tau$$\sim$230 ns, if a more open Mb conformation is a result of sol-gel encapsulation the CO escape rate should be faster than $\tau$$\sim$960ns. The difference suggests viscosity effects may be a factor between solution and sol-gel results. However, previous studies on PRODAN encapsulated sol-gels utilizing steady-state emission anisotropy and time-resolved emission techniques found that the local viscosity of the pores, at the age time examined here, display a viscosity of 2cP. The viscosity of water at room temperature is 1.002cP. In addition, CO rebinding is highly viscosity dependent and the second order rate constant calculated from PBD for CO rebinding to Mbsol-gels are similar to those under solution conditions. Thus, a pure viscosity effect is not likely the reason for the observed results.

Interactions between the protein exterior and negatively charged sol-gel pore have been previously discussed as a contributing factor in sol-gel studies. A more open conformation may be a direct result of electrostatic interactions between the protein tertiary structure and the lining of the sol-gel pore. Gottfried and Authors demonstrated that sol-gel conditions, like the ones here, decreased rotational freedom of encapsulated MgMb. The observed increase in rotational correlation time was attributed to
increased local viscosity, but they also consider covalent and non-covalent interactions between the sol-gel and the protein exterior as the cause of restricted rotational movement. Interestingly, a study on the pH dependence of interaction between negatively charged lipids and Mb found that Mb is negatively charged at pH 7 and that Mb is not positively charged until pH 4. Thus electrostatic interactions do not appear to be a main contribution to the observed differences.

If sol-gel confinement promotes a more open conformation the increase CO exit rate may be due to increase in solvent shell stabilization induced by the SiO\(^{-}\) groups lining the cavity. Stabilization would limit solvent reorganization between transients that require a change in hydration pattern. Thus transients necessary for CO escape may require a change in solvent organization. The effect of increased water stabilization was illustrated in a study probing the conformational dynamics of encapsulated R and T state hemoglobin (Hb). The significant slowing of relaxation processes between R and T state Hb at 10ºC was suggestive of immobilization of the hydration shell. Furthermore, Samuni demonstrated the ability to trap spectroscopic and kinetically different Mb species in sol-gels that maintained the COMb or deoxyMb equilibrium conformations subsequent to CO release or rebinding, respectively. Interestingly, while differences in enthalpy, volume changes, and kinetics of the protein dynamics are observed, sol-gel encapsulation still permits effective protein function.

### 3.6 Future Direction

Finally, all of the work shown here proves that these methods can be utilized with solid state materials and will provide us with another tool to investigate the effects of confinement on biomolecules. Now that the ground work has been laid for investigating COhhMb sol-gels with time-resolved photothermal methods, further measurements on
viscosity, ionic strength and pH dependence would aid in unraveling the complete effects of confinement.

In order to elucidate if encapsulation does perturbs the tertiary structure of Mb to a more open conformation similar to pH 3.5 solution, the same experiments should be performed on Mbsol-gels formed in pH 3.5 buffer and formed in pH 6.5 buffer than soaked in pH 3.5 buffer. A comparison between these protocols may reveal the true nature of the dynamics associated with CO escape. In addition, the true effect of viscosity can be probed with conducting PAC and PBD measurements on the same Mbsol-gel as a function of aging time, i.e. 12hrs to days after the gel is formed. This does pose a problem in that the gels shrink with time, therefore great lengths should be taken when collecting data to ensure the gel does not physically move. And finally, a comparison between time-resolved thermodynamics of Mb and horseradish peroxidase encapsulated in sol-gel will help in understanding the effects of confinement on proteins which require large protein fluctuations for ligand exit (Mb) and those that have an open access channel (HRP) with minimal energetic barriers for ligand exit.

3.7 References


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Chapter 4: System II-Zirconium Phosphate

4.1 Background.

The demand for novel functional materials with novel catalytic and sensing properties has stimulated research in the area of solid state host-guest chemistry. This involves the integration of inorganic or organic complexes into rigid solid state frameworks to form robust materials exhibiting diverse functionality.\textsuperscript{1-4} One type of inorganic framework commonly used as a solid state host are zirconium phosphates. Zirconium phosphates are clay like layered materials that are chemically robust, easily synthesized under relatively mild conditions and can serve as good ion exchangers. Zirconium bis (monohydrogen orthophosphate) monohydrate $\text{Zr(HPO}_4\text{)}_2\cdot\text{H}_2\text{O}$, also known as $\alpha$-zirconium phosphate($\alpha$ZrP) consists of a zirconium atom plane with three of the phosphate oxygens bound to three different Zr ions. The fourth phosphate oxygen is an easily ionizable OH directed away from the Zr plane into the interlamellar space and the bulk solution. This form of zirconium phosphate has one water molecule per molecular unit and an interlayer distance of 7.6 Å. This distance can expand with the exchange of cationic organic guest molecules to allow for intercalation.

Figure 4.1 Structure of expanded $\alpha$ZrP The ZrP structure was obtained from the Crystallographic Data Base.
of larger molecules such as fluorescent probes, porphyrins and even proteins. For example, butylammonium exchanged ZrP (BAZrP) produces an interlayer distance of 18.6 Å. BAZrP has been used to intercalate myoglobin, lysozymes, hemoglobin, chymotrypsin, and glucose oxidase to study structure and activity after immobilization. The successful intercalation of these proteins was demonstrated by a large increase in interlayer distance. Interestingly, intercalation did not significantly alter the enzyme activity and in some cases a slight increase in activity was observed. These materials have been utilized as bio-catalyst by intercalating proteins such as hemoglobin (HbA), myoglobin (Mb), horseradish peroxidase (HRP), lysozyme and glucose oxidase. These materials show enhanced spacing of the αZrP layers (due to protein intercalation) and retention of the secondary structure for the intercalated proteins. The peroxidase activity observed for HbA, Mb and HRP in aqueous buffer solutions is also apparent upon intercalation although the overall catalytic activity is reduced.

Slight modifications to the synthesis of αZrP produces a hydrated form, with six water molecules per molecular unit and an interlayer distance of 10.3 Å (ZrP-H). Colon et al. has directly ion exchanged Ru(II)tris(2,2’ bipyridine) in between the layers of a ZrP-H without the aid of cationic organic ‘spacers’. The intercalation of Ru(II)tris(2,2’ bipyridine) was confirmed by the expansion of interlayer spacing to 15.2 Å. The resulting Ru(II)tris(2,2’ bipyridine): ZrP-H complex exhibits a hypsochromic shift in the emission spectrum along with an increase in emission intensity.

4.2 Photophysics of free-base 5,10,15,20-tetrakis(N-methylpyridium-4-yl) porphyrin adsorbed in expanded αZrP.
Porphyrins represent diverse class of macromolecules with unique catalytic and spectroscopic properties making them attractive candidates as guest molecules in layered materials. Free base porphyrins display absorption bands covering a wide region of the visible spectrum with high extinction coefficients as well as relatively high emission quantum yields, long singlet state lifetimes and high inter-system crossing probabilities resulting in high quantum yields for triplet state formation\(^{(11-12)}\). These properties are ideal for a wide range of applications including optical sensing, photochemistry and photodynamic cancer therapy to name only a few. The porphyrin ring also serves as a ligand for a wide range of metals expanding the versatility of porphyrin chemistry. For example, iron porphyrins (i.e., heme) are widely exploited in nature to perform a plethora of critical biochemical process ranging from oxygenation of organic substrates (heme oxygenases), electron transfer (cytochromes), bioenergetics (heme-copper oxidases) and even cellular signaling (e.g., FixL, Globin Coupled Sensors, etc)\(^{(13-16)}\). Manganese porphyrins have also been widely utilized as catalysts for oxygenation reactions.

With this in mind, porphyrins and metalloporphyrins have been encapsulated into a wide range of solid state materials including clays, zeolites, sol gels and Nafion membranes\(^{(17-25)}\). Porphyrins have also been utilized to construct extended porous frameworks\(^{26}\). In terms of clays and clay like materials, the porphyrin of interest in this study, meso-tetrakis(N-methylpyridinium-4-yl) porphyrin(TMPyP) (Figure 4.2), has been
adsorbed on laponite, sumecton, layered silicates and modified ZrP\(^{(17-22)}\). TMPyP is attractive to host guest chemistry since the porphyrin ring contains four cationic pyridyl groups that are nearly perpendicular to the porphine plane. These charged groups allow for adsorption of the porphyrin onto charged matricies through electrostatic interactions. Previous studies have found that significant spectroscopic changes can arise from a rotation of the porphyrins pyridium groups and/or porphyrin ring planarity thus making this porphyrin a sensitive environmental probe.\(^{17-22,26}\) For example, Cherna and Gill observed both 30nm and 60nm bathochromic shifts in the spectrum for TMPyP adsorbed on the outer surface and in between the layers of lamponite respectively, which they attribute to the rotational hindrance of the TMPyP pyridyl groups.\(^{17}\) Similarly, TMPyP ion exchanged in silicates resulted in bathochromic shifts in the absorption spectra and significant quenching of the fluorescence. Čeklovský et al. determined that flattening of the four pyridal groups was the cause of the observed spectral shifts, and that quenching was due to the formation of a porphyrin bilayer in the interlayer spacing\(^{18}\). In a more recent report they also state that observed multiphasic fluorescence lifetimes can be attributed to multiple populations with varying TMPyP intermolecular interactions as well as populations of isolated porphyrins.\(^{19}\)

Interestingly, despite the advantageous properties of the cationic TMPyP described above, few studies have examined the mechanisms of association between TMPyP and the 10.3Å ZrP layered materials. There have been experimental and molecular modeling studies focused on the interlayer state of TMPyP exchanged \(\alpha\)ZrP with the assistance of organic spacers. Li et al. found that the preferred arrangement for intercalated TMPyP in \(\alpha\)ZrP is a tilted monolayer without aggregation and that BA modified \(\alpha\)ZrP provides the fastest mode of intercalation.\(^{28,29}\) In the present study we examine the photophysical properties of TMPyP directly ion exchanged into ZrP-H as
well as absorbed onto αZrP galleries without organic modifications. The photophysical properties, including optical absorption and both steady state and time resolved emission data are powerful probes for examining the modes of adsorption as well as the chemical environment of the bound macrocycle.

4.2.1 Materials and Methods. All reagents were purchased from Sigma-Aldrich and used without further purification. The dense phase αZrP was prepared by adding 50 mL of 0.5 M ZrOCl₂ · 8H₂O solution slowly to 100mL of 6 M H₃PO₃ at 90°C with constant stirring. This temperature was maintained for 48 hrs. The resulting solid was washed with several times with deionized water. To prepare the TMPyP exchanged αZrP a small amount of this material (~1mg) was sonicated in a solution of 1mM TMPyP for 10 minutes (TMPyP-αZrP). After 1hr of incubation the resulting green material was washed several times with deionized water and air dried. X-ray powder diffraction (XPD) (Bruker AXS D8 Advance θ/2θ Diffractometer) was used to characterize the inter-layer spacing of the material.

10.3 Å ZrP(ZrPH) was synthesized following a procedure described previously⁵. Briefly, 50 mL of a 0.5 M ZrOCl₂ · 8H₂O solution was added drop wise over a period of 1hr into 100mL of 6 M H₃PO₃ at 94°C with constant stirring. The reaction mixture was then refluxed at 94°C for 5 days after which the precipitate was centrifuged and washed several times with deionized water. The ZrPH material was confirmed from XPD data of the wet sample. This material was kept hydrated, as drying causes the material to collapse to the αZrP form. For synthesis of the low loading TMPyP:ZrP material (LowTMPyP-ZrPH) ~100 mg of ZrPH was sonicated for 10 min. in a solution of 1 uM TMPyP. After 24 hrs the green material was centrifuged and washed several times with deionized water until negligible amounts of TMPyP were detected in the supernatant.
The same procedure was followed for high loading of TMPyP with the exception that 
~100 mg of ZrPH was sonicated for 10 min in a 1 mM TMPyP solution (high TMPyP- 
ZrPH). Both high and low loading TMPyP-ZrPHs were air dried for analysis.

All solution Uv/Vis spectra were obtained using a Shimadzu UV2401 
spectrometer. An ISS PC1 spectrofluorimeter was used in collecting steady-state 
fluorescence data. A small amount of powder sample was mounted on a glass slide 
using a thin layer of vacuum grease. The glass slide was placed at a 57° angle relative 
to the excitation source in the PC1 sample holder. Time-resolved fluorescence decays 
were collected on a home built instrument. The glass slides containing samples were 
placed in a 1cm sample holder oriented 57° relative to the excitation pulse. The 
excitation pulse was derived from a Continuum Leopard II frequency doubled Nd:YAG 
laser (< 20 ps pulse width, 532 nm, 20 Hz, ~30 mJ/pulse). The emitted light was passed 
through a focusing lens followed by a long pass filter and then onto the face of an 
Electro-Optics Inc., EOT 2030 amplified Si diode (300 ps rise/fall time). The resulting 
signal was digitized using a Tecktronix TDS7404 4 GHz DPO.

4.2.2 Results The absorption spectra of TMPyP in aqueous solution, low 
TMPyP-ZrPH and highTMPyP-ZrPH are shown in Figure 2. The Soret absorption 
maxima are centered at 421nm, 428nm, and 439nm for TMPyP in aqueous solution, low 
loading and high-loading TMPyP-ZrPH respectively. The corresponding emission 
spectra (using Soret excitation) are displayed in Figure 4.3. TMPyP in aqueous solutions 
exhibits a broad emission band center at ~ 686nm while the emission spectra of the 
TMPyP-ZrPH materials exhibit two additional bands centered at ~670nm and ~714nm. 
Figure 4.3 provides an overlay of corresponding lifetime data for TMPyP in solution, low-
and high-ZrP-H. The singlet excited state decay of TMPyP in water is monophasic with
a lifetime of ~5.4ns, while the singlet state decay of the adsorbed TMPyP display is biphasic with emission lifetimes of 5.7ns and 1.9ns corresponding to 49% and 51% of the total intensity, respectively. At low loadings the TMPyP-ZrPH material exhibits a slow phase of ~8.4ns accounting for 5% of the population and a fast phase of ~1.17ns accounting for the remaining 95%, while at high loadings a slow phase of ~2.3ns (37% of total population) and a fast phase of ~0.6ns (63% of the total population) are observed. Polarization measurements of TMPyP in aqueous solution as well as low and high loading TMPyP-ZrPH are found to be 0.001±0.006, 0.107±0.004, and 0.678±0.09 respectively. Table I provides a summary of the spectral results.

Figure 4.3: (Left) Normalized absorption spectra of TMPyP in an aqueous solution (solid line), low loading TMPyP-ZrPH (dotted line), and high loading TMPyP-ZrPH (dashed line). (Right) Normalized emission spectra of TMPyP in an aqueous solution (dashed line), low loading TMPyP-ZrPH (solid line), high loading TMPyP-ZrPH (green line), and TMPyP-αZrP (dash-dotted line).
Figure 4.4 displays an overlay of the XPD patterns for wet expanded ZrP-H, as well as for dry high- and low-loading TMPyP-ZrP materials. The low angle diffraction peak confirms an interlayer distance of 10.3 Å for ZrP-H. After dehydration, the same material collapses to the dense form of ZrP, resulting in a decrease interlayer spacing to 7.3 Å. When hydrated ZrP is sonicated with low concentrations of TMPyP in aqueous solutions, washed and dried the resulting material is green in color verifying bound TMPyP however the XRD pattern reveals an interlayer spacing of 7.3 Å (Figure 4.4, solid line top). Therefore, it is not possible to have intercalated TMPyP. If the same treatment of expanded ZrP is performed with high concentrations of TMPyP the resulting XRD

<table>
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<th>Sample</th>
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<th>$A_s$</th>
<th>$\tau_f$</th>
<th>$A_f$</th>
<th>Polarization</th>
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<td>TMPyP-H2O</td>
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<td>1.0</td>
<td>-</td>
<td>-</td>
<td>0.001± 0.006</td>
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<tr>
<td>TMPyP-αZrP</td>
<td>5.7</td>
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<td>1.9</td>
<td>0.51</td>
<td>0.112±0.004</td>
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<tr>
<td>Low loading ZrP-H</td>
<td>8.4</td>
<td>0.05</td>
<td>1.17</td>
<td>0.95</td>
<td>0.42± 0.02</td>
</tr>
<tr>
<td>High loading ZrP-H</td>
<td>2.3</td>
<td>0.37</td>
<td>0.6</td>
<td>0.63</td>
<td>0.68± 0.09</td>
</tr>
</tbody>
</table>

Figure 4.4: X-ray powder diffraction data. Left: Dried ZrPH (dotted line) and dried low loading TMPyP-ZrPH (solid line) right: dried ZrPH (dotted line) and dried high loading TMPyP-ZrPH(solid line)

Table 4.1: Summary of emission lifetime and polarization results for TMPyP. Lifetimes are in ns and amplitudes are fraction of total emission.
pattern (Figure 4.4 solid line bottom) shows a significant increase in interlayer spacing to 12.2 Å indicating intercalation.

4.2.2.1 Electronic Absorption:

In solution, TMPyP exhibits a Soret maxima at 421nm that is bathochromically shifted when absorbed onto ZrPH. The observed shifts are larger for high loading TMPyP-ZrPH (20nm shift) than for low loading TMPyP-ZrPH (10nm shift) indicating that while the bathochromic shifts are caused by intermolecular interactions between the porphyrin and material framework, the origin of the shift is distinct between the two materials. This is further demonstrated by the fluorescence lifetimes. When comparing the TMPyP-ZrPH materials to TMPyP in water the emission lifetimes associated with the materials become biphasic indicating more than one population of bound TMPyP. However the magnitude and lifetimes of these populations are distinct for each material. These differences can be rationalized in terms of intercalation and/or surface adsorption of the porphyrin, with the different modes of binding possessing distinct chemical environments.

With respect to changes in the absorption spectra, previous studies attribute bathochromic shifts in TMPyP absorption spectra to polarity effects of the solvent, the protonation state of the porphyrin ring nitrogens, aggregation of the porphyrins and/or changes in the torsion angle of the methyl pyridium groups.\textsuperscript{18-22} In the case of solvent effects, large changes in the dielectric constant/polarity typically result in 1-2nm shifts in the absorption maxima which are much smaller than what is observed here.\textsuperscript{12,25,26,28} Thus the observed absorption shifts are not simply due to a high dielectric constant environment. In addition, the pKa value for TMPyP is less than 1.2 which is considerably lower than most porphyrins due to the methyl pyridyl moieties serving as strong electron-
withdrawing groups. Therefore a highly acidic environment would be needed to protonate the porphyrin ring nitrogens. Protonation of the interior nitrogens also increases the symmetry from \( D_{2h} \) to \( D_{4h} \) which significantly influences the porphyrin Q bands due to vibronic coupling of the two transitions.\(^{18,28,29} \) In the case of both low and high loading TMPyP-ZrPH the visible bands are consistent with \( D_{2h} \) symmetry suggesting the interior nitrogens are deprotonated in the materials.

Porphyrin aggregation on the surface of ZrPH is also a possible mechanism accounting for the large bathochromic shift in the absorption bands. Dipole coupling of porphyrins parallel to one another forms two types of aggregates each with different electronic and spectroscopic signatures. H-type aggregates have transitions to higher energy states relative to the monomer, resulting in a hypsochromic shift in the absorption spectra and quenching of the fluorescence.\(^{26} \) Since adsorption of TMPyP resulted in shifts to lower energy, H-type aggregates are unlikely. On the other hand, J-type aggregates have transitions lower in energy characterized by a bathochromic shift in the absorption spectra as well as a very small stokes shift in the emission spectrum.\(^{26} \) Although a bathochromic shift is observed in the absorption spectra for both TMPyPZrPH materials the large stokes shifts observed for both materials (~30 nm) preclude formation of J-type aggregates upon adsorption.

Rotation of the pyridyl groups associated with the porphyrin periphery to a more planar geometry is, however, consistent with the observed spectroscopic data. Crystallographic studies as well as geometry optimization calculations demonstrate that the lowest energy conformation of the prophyrin is that in which the pyridium groups are nearly perpendicular to the porphine ring.\(^{17} \) Rotation of pyridyl rings towards a coplanar conformation increases resonance interactions between the \( \pi \) systems of both the
porphyrin parent ring and the peripheral pyridyl group.\textsuperscript{29} This results in a shift in electron density from the porphyrin core towards the four positively charged pyriduim groups\textsuperscript{18} resulting in a bathochrmic shift in the absorption spectrum. Theoretical results by Chernia and Gill have shown that a $\sim$30nm shift in the porphyrin absorption occurs with a change in pyridyl group dihedral angle (relative to the porphyrin plane) by $\sim$60°.\textsuperscript{18} The differences in the magnitude of the bathochromic shift between the low and high loading materials can be explained by heterogeneity in the modes of binding. Consequentially, adsorption of TMPyP under high loading conditions must be to binding sites in which interactions between the porphyrin and ZrPH increase the rigidity of the porphyrin or a flattening of the porphyrin ring. In contrast, the interactions between TMPyP and ZrPH under low loading conditions must result in torsion of the pyridium groups but not to the extent of the highTMPyP-ZrPH (evident by a smaller bathochromic shift).

4.2.2.2 Steady State Emission and Excited State Lifetimes:

The emission spectra and excited state lifetimes are also consistent with conformational perturbations to the porphyrin ring upon adsorption to ZrPH. TMPyP in aqueous solution exhibits a broad emission band centered at 686nm which arises from an intramolecular CT state in which one electron is transferred from the porphine ring to a pyridinium group and a nearby S1 exited state. The CT state has been previously calculated to be $\sim$2.0 eV, nearly equal to the 1.9 eV for the S1 excited state.\textsuperscript{27} Upon excitation of TMPyP in solution there is rapid rotation of the pyridinium groups to a coplanar position which increases conjugation of the $\pi$ systems resulting in featureless emission spectrum. Rotational hindrance of the pyridinium methyl groups decrease resonance interactions between the ring systems reducing the strong mixing of the CT
and S1 states resulting in presence of two bands in the emission spectrum. The adsorption of TMPyP to ZrPH materials gives rise to an emission spectra with at least two bands. This indicates that at least two populations of TMPyP exists, with at least one population resulting from restricted motion of the pyridyl groups. The presence of two populations of emitters is also supported by the biphasic nature of the emission decays for the TMPyP-ZrPH materials. These populations are likely to consist of TMPyP molecules adsorbed onto the surface of ZrPH as well as those intercalated between the layers of ZrPH (see Figure 4.5).

The clear differences in the emission lifetimes and polarization for each of the materials (Table 1) are also consistent with heterogeneous association between the porphyrin and ZrP (i.e., intercalation and/or surface adsorption). The TMPyP chromophore in H2O exhibits a monophasic emission decay with a lifetime of ~5.4ns and polarization of 0.001± 0.006 consistent with free rotation of the TMPyP in solution as well as the pyridyl groups adopting an orientation nearly perpendicular to the porphyrin plane. In contrast, association of TMPyP with ZrP results in an increase in the emission polarization in the order: TMPyP-αZrP < LowTMPyP- ZrPH< HighTMPyP-ZrPH as well as biphasic emission decays.

4.2.2.3 Potential Modes of Binding:

The results presented above can be rationalized in terms of different modes of binding of TMPyP to the ZrP depending upon the physical nature of the material. Consider first the TMPyP-αZrP material with a polarization of 0.112 ± 0.004 and biphasic emission decay with lifetimes of ~5.7ns and ~1.9ns (49% and 51% of the total intensity respectively). The population of emitters with τ < 5.7ns are likely to experience an environment similar to the porphyrin in solution in which the peripheral pyridyl groups are
unperturbed. It is possible to have electrostatic interactions between the surface hydroxyl groups of αZrP and the positively charged nitrogen on one of the substituent groups (Figure 4.5, mode A). This would allow the porphyrin free rotation about the pyridyl group as well as free rotation of the remaining unbound pyridinium groups analogous to the porphyrin in solution. The second porphyrin population with a lifetime ~1.9 ns is also adsorbed to the surface of αZrP but not intercalated (i.e., no shift in the XRD diffraction peaks, Figure 4.4). This population may be one in which the porphyrin is adsorbed parallel to the surface hydroxyl groups (Figure 7, mode B). This would allow electrostatic interactions between the negatively charged surface of the material and multiple positively charged porphyrin pyridyl groups. The large increase in polarization in the absence of a large decrease in lifetime for TMPyP-αZrP suggest that the porphyrin exhibits restricted motion upon adsorption. The singlet excited state lifetime difference for the parallel bound porphyrins may be attributed to an increase in vibronic coupling between the porphyrin and ZrP matrix after excitation resulting in differences in non-radiative decay rate constants. i.e., $k_{nr}$ mode B > $k_{nr}$ mode A.

For the low loading TMPyP-ZrPH the results indicate an increase in the polarization to $0.42 \pm 0.02$, an increase in the slow phase fluorescence lifetimes (~8.5 ns) and a small decrease in the fast lifetime (~1.7 ns). Note however that the slow lifetime makes up only 5% of the total intensity and the magnitude suggest that this population may be equivalent to the population associated with mode A for the TMPyP-αZrP. It is evident from the XPD data that TMPyP is also not intercalated between the layers of ZrPH under low loading conditions. Thus structural differences between the αZrP and hydrated αZrP result in a shift in the equilibrium distribution towards the population associated with mode B with a 1.7ns lifetime (accounting for 95% of the
adsorbed porphyrin). The increase in this population would also result in an increase in fluorescence polarization as observed.

Lastly, the high loading TMPyP-ZrPH exhibits a polarization of 0.68±0.09 with singlet excited state lifetimes of ~2.3ns and ~0.6ns (37% and 63% of the total intensity respectively). In contrast to the above materials, the XRD data for the high loading TMPyP-ZrPH confirms that one mode of binding is intercalation between the layers of ZrPH (binding mode C in figure 4.5c). Binding mode C is comparable to binding mode B except that the ZrPH layers now occupy both sides of the porphyrin plane. This results in enhanced non-radiative decay of the TMPyP singlet excited (i.e., \( k_{nr} \) modeC > \( k_{nr} \) modeB > \( k_{nr} \) modeA) giving rise to the ~0.6ns lifetime. This population of bound TMPyP accounts 63% of the total population of emitters. The remaining 37% is surface bound with similar excited state decay parameters as mode B in the low loading ZrPH and \( \alpha ZrP \) materials.

Figure 4.5: Schematic diagram illustrating possible TMPyP binding modes when adsorbed onto ZrP-H
4.2.3 Conclusions. The results here demonstrate multiple modes of binding of the cationic TMPyP onto ZrPH gallaries by way of ion exchange. The modes of binding include two modes of surface adsorption as well as intercalation depending upon the nature of the material (hydrated or dense forms of ZrP) and the relative concentrations of TMPyP present during adsorption. The photophysical properties suggest that the environment of the adsorbed TMPyP is heterogeneous for each of the materials. Intercalation of TMPyP between the layers of ZrPH requires high concentrations of porphyrin and is confirmed by XPD. The interlayer distance suggests a slightly tilted porphyrin monolayer. The excited state properties further reveal that the interactions between ZrPH and TMPyP affect the conformations of the four cationic pyridium groups relative to the porphyrin plane. Bathochromic shifts in the absorption spectra and the resolution of three bands in the fluorescence spectra can all be attributed to rotation of the substituents pyridyl rings to a more coplanar confirmation.

4.3 Excited State Properties of 9-Amino Acridine Surface Adsorbed onto αZr-Phosphate Galleries

The acridines (including 9-amino acridine or 9AA) have been widely explored as an environmentally sensitive fluorescent probe and thus can be exploited as a fluorescent guest sensor.

Figure 4.6 9 amino acridine

1 Portions of these results have been previously published (Vetromile et al 2011) and are utilized with permission from the publisher.
In this report we examine the photophysical properties of 9AA intercalated into αZrP for potential use in (host-guest)-guest sensor applications.

4.3.1 Methods and Materials. All reagents were purchased from Sigma-Aldrich and used without further purification. The αZrP was prepared by dissolving 1.5 g of ZrOCl$_2$•8H$_2$O in 50 mL of deionized water. To this solution was added 100 mL of 42% (by weight) H$_3$PO$_4$ dropwise with rapid stirring. This mixture was brought to 90°C and maintained at this temperature for 48 hrs. The resulting solid material was collected via centrifugation (5,000xg), washed three times with deionized water and air dried. The resulting solid material was characterized using X-ray powder diffraction (XPD) (Bruker AXS D8 Advance θ/2θ Diffractometer). The 9AA-αZrP material was prepared by sonicating ~ 100 mg of αZrP in the presence of 5 mL of ethanol containing 1 mM 9AA. This solution was then incubated for 48 hrs followed by centrifugation (3,000xg). The solid material was washed four times with ethanol and air dried.

In order to determine the loading of 9AA, 34.5 mg of dried 9AA-ZrP was sonicated in 1 mL of ethanol for 2 minutes. To determine the number of 9AA molecules per αZrP unit, 5 μL of the suspension was diluted into 2 mL of ethanol in a 1 cm quartz cuvette and the UV-Vis absorption spectrum obtained. The spectra were corrected for scatter by fitting a narrow spectral region to a linear function. This scattering baseline was then subtracted from the entire spectrum. The concentration of 9AA in the cuvette was calculated using the corrected absorption at 402 nm and molar extinction coefficient, ε, of 13 mM$^{-1}$ cm$^{-1}$. From this, the number of 9AA molecules was determined using Avogadro’s number, N, and the total volume of solution. The weight of solid in the cuvette was converted to units of αZrP using 190 g/unit-mol and Avogadro’s number. The number of 9AA molecules is then divided by the number of αZrP units and reported as...
9AA loading. The theoretical loading was determined by assuming that the 9AA ring system lies parallel to the surface of αZrP gallery. This would predict the largest surface area that the molecule could occupy on the αZrP unit and the lowest theoretical loading. Atom to atom distances were used to determine a surface area of 55.1 Å² for 9AA (i.e., 9.13 Å² along the long access and 6.03 Å² along the short axis). The surface area of one unit of αZrP is 24 Å², giving a theoretical loading of 0.435 9AA molecules per unit of αZrP. 

Solution UV/Vis spectra were recorded using a Shimadzu UV2401 spectrometer while solid-state absorption spectra were obtained using a Perkin-Elmer Lambda 900 Uv/Vis/NIR spectrophotometer. Steady-state fluorescence measurements were carried out using an ISS PC1 spectrofluorimeter. The samples were placed on small glass cover slips using a thin layer of vacuum grease. Approximately 5 mg of material was used. The samples were then placed in the PC1 sample holder at a 57° angle relative to the excitation source. The samples were excited at 423 nm and the emission monochromator scanned from 440 to 600 nm. Time-resolved fluorescence decays were obtained by placing the samples as described above into a 1-cm sample holder in the optical path of our home-built fluorescence lifetime instrument. The samples were oriented 57° to the excitation pulse. The excitation pulse was derived from a Continuum Leopard II frequency tripled Nd:YAG laser (< 20 ps pulse width, 355 nm, 20 Hz, ~ 30 mJ/pulse). The emitted light was passed through a focusing lens, through a 350 nm low-pass optical filter, and onto the face of an Electro-Optics Inc., EOT 2030 amplified Si Diode (300 ps rise/fall time). The resulting signal was digitized using a Tecktronix TDS7404 4 GHz DPO. Traces are the average of ~20 laser pulses.
4.3.2 Results

An overlay of the absorption spectrum of 9AA in ethanol and that of the 9AA-ZrP is displayed in Figure 4.7. Interestingly, both spectra display major absorption bands at 383 nm, 402 nm and 425 nm although the band width is much larger in the case of the 9AA-ZrP material. The corresponding emission spectrum
(excited at 423 nm) of 9AA in ethanol exhibits maxima centered at 455 nm and 483 nm with a small shoulder at 520 nm. The emission spectrum of the 9AA-ZrP material displays broad bands centered at ~462 nm and ~490 nm with a weaker band centered at 520 nm. The emission lifetimes are also effected by adsorption (Figure 4.7). In ethanol 9AA exhibits a monophasic singlet state decay with a lifetime of 16.5±0.02 ns while the decay becomes biphasic when adsorbed onto either αZrP with lifetimes of 1.6±0.1 ns (57 % of the total intensity) and 9.8±0.5 ns (43% of the total intensity) for 9AA-ZrP.

The difference in both the steady state emission spectra as well as emission lifetimes associated with the 9AA-ZrP material, relative to 9AA in ethanolic solution, indicate significant intermolecular interactions between the αZrP material and the 9AA chromophore. In addition, the biphasic emission decay further indicates at least two populations of adsorbed 9AA in the αZrP material. Such heterogeneous populations could be associated with multiple binding modes on the ZrP surfaces, multiple binding modes associated with intercalation, and/or binding to both the surface as well as intercalation. The XRD data for the 9AA-ZrP material (Figure 4.8) does not indicate intercalation since the low angle scattering peak at 2θ = 12 does not shift upon incubation with 9AA indicating no increase in the average interlayer spacing. Since the average distance between layers in this material is ~ 7.4 Å (the dense phase distance of the ZrP samples prepared in this work) and the minimum van der Waals distance for 9AA is ~3.5 Å (i.e.,
van der Waals radius of carbon and considering an intercalation mode in which the planar face of the 9AA is parallel with the planar faces of the ZrP) a 2θ ~ 8 would be expected for intercalation.37

Table 4.2 Summary of loading calculations for 9AA-ZrP

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<td>8.2 ± 0.9 x 10⁻³</td>
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</tbody>
</table>

Previous studies of acridine intercalated into αZrP-NBA shows concentration dependent emission intensity and lifetime changes that were attributed to strong intermolecular interactions at high loadings.38,39 The intermolecular interactions are evident from both the appearance of a long wavelength absorption band and a hypsochromic shift in the emission spectrum which arise from a decrease in distance between bound acridine molecules. At lower loadings the spectral properties of the bound acridine (The Authors estimated that the acridine was 75-80% under these conditions) were identical to those in solution indicating that the bound acridine was monodispersed with little intermolecular interactions. In the case of the 9AA-ZrP material no significant shift is observed in the optical spectrum and bathochromic shifts are
observed in the emission spectrum (i.e., no excimer bands are observed). This is consistent with the loading values obtained for the adsorption of 9AA onto the αZrP which indicate ~2% of the surface area of the ZrP gallery is occupied by 9AA molecules (see Table 4.2). In contrast, incubation of αZrP in solutions of 5 mM 9AA produce a material with significant excimer emission (Figure 4.9) and significantly shortened emission lifetimes (fast phase decay of ~30 ps and a slow phase of ~6 ns, data not shown) indicating inter-molecular interactions between the bound 9AA. Thus, it is clear that under the lower loading conditions, 9AA adsorption is monodispersed with little intermolecular interactions between 9AA molecules.

![Figure 4.10](image)

**Figure 4.10** Steady-state emission spectra of 9AA in 75/25 (V/V) ethanol/water pH 3 (solid line) and 75/25 (V/V) ethanol/water pH 9 (dotted line). Inset: 9AA-ZrP incubated in water at pH 6 (solid line) and pH11 (dotted line).

The spectral properties of the 9AA chromophore are also sensitive to pH. In solution at low pH (< 4) the emission spectrum of 9AA exhibits well resolved bands centered at 433 nm, 456 nm and 484 nm while at a pH > 9.5 a broad band is observed
centered at 456 nm with shoulders at 467 nm and ~490 nm (Figure 4.10). The fact that the emission spectrum of the bound 9AA resembles that of 9AA solubilized in ethanol suggests that the 9-amino group remains predominately deprotonated upon binding to the ZrP despite the acidic nature of the material. In addition, examination of the steady state emission of the 9AA-ZrP material in water at pH 6 or pH 11 reveals only slight shifts in the emission maxima (Figure 4.10, inset). This is further evident from the emission lifetimes (Table 4.2). For 9AA in acidic ethanol solution (pH < 3.5) the emission lifetime is 22 ns while at pHs > 9.5 the lifetime decreases to 19 ns (data not shown). The fact that the lifetime of the 9AA-ZrP material is significantly shorter than the free 9AA in either acidic or basic solution conditions suggests that the protonation state of the 9AA is not affected by the acidic nature of the ZrP.

### Table 4.3 Summary of the emission lifetime results for 9AA. Lifetimes are in ns and amplitudes are percent total emission

<table>
<thead>
<tr>
<th>Species</th>
<th>$t_1$</th>
<th>$A_1$</th>
<th>$t_2$</th>
<th>$A_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9AA in Ethanol</td>
<td>16.5 ±0.2</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9AA, pH &lt;3.5</td>
<td>22±0.1</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9AA, pH &gt;9.5</td>
<td>19±0.1</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9AA-ZrP</td>
<td>1.6±0.1</td>
<td>75</td>
<td>9.8±0.5</td>
<td>25</td>
</tr>
</tbody>
</table>

The spectral properties of the 9AA-ZrP material can be interpreted using the photophysics of the parent acridine chromophore. Acridine, like other N-heterocyclic molecules, exhibits two closely spaced excited states which can be described as $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ in character. The excited state properties, including the emission maxima and emission lifetimes depend upon the relative positioning of these two states. Two mechanisms have been suggested for the relative positioning of these states with the first involving solvent interactions with the N-atom of the heterocycle which can stabilize/destabilize the $n\rightarrow\pi^*$ state and the second involving vibronic coupling between
the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ states which is modulated by out of plane vibrational modes.\(^{39}\)

Within both mechanisms solvation of the acridine ring systems in non-hydrogen bonding solvents results in the excited state being primarily of $n \rightarrow \pi^*$ character which leads to rapid non-radiative decay via intersystem crossing and vibrational relaxation.\(^{40,41}\) In contrast, for strongly hydrogen bonding solvents the lone pair electrons on the pyridyl nitrogen atom become stabilized via hydrogen bonding with solvent molecules thus lowering the energy of the excited state with $\pi \rightarrow \pi^*$ character below the $n \rightarrow \pi^*$ state. This increases the rate of radiative relaxation (increases the fluorescence quantum yield) and produces bathochromic shifts in the emission spectrum, relative to the chromophore in non-hydrogen bonding solvents. Thus, in progressing from hydrogen bonding to non-hydrogen bonding environments the observed emission lifetime decreases and the emission maximum becomes increasingly hypsochromically shifted.

In the case of the 9AA-ZrP the absorption spectrum exhibits similar transition energies as 9AA in ethanol while the emission maxima are bathochromically shifted and the emission lifetime becomes shorter and biphasic.

Within the context of acridine photophysics the bathochromic shift is consistent with 9AA
adsorbed onto the surface of the ZrP galleries in a manner that facilitates hydrogen bonding between the pyridyl N-atom and surface bound OH groups (figure 4.11). The observed lifetime, however, is shorter than what would be expected for H-bonded 9AA and is biphasic suggesting additional mechanisms for excited state deactivation (i.e., deactivation of the π→π* state) are present in the 9AA-ZrP materials. Such mechanisms (Figure 4.12) include resonance energy transfer, photo-induced electron transfer, enhanced inter-system crossing (ISC) and enhanced energy transfer from the excited singlet state to the solvent bath. Resonance energy transfer is obviously not possible since the ZrP exhibits no absorbance in the region of the 9AA emission. In addition, photo-induced electron transfer is also not likely due to the low redox potential of the ZrP framework.42

![Figure 4.12](image)

**Figure 4.12** Schematic diagram of the excited state transitions of the 9AA in ethanol and associated with αZrP. Solid arrows are radiative decay pathways and dashed arrows are non-radiative decay pathways.
In the case of ISC, the rate (i.e., singlet to triplet state conversion) is dependent upon the spin-orbit coupling between the states according to:

$$\langle \Psi_{S(i,\pi^*)} | H_{so} | \Psi_{T(i,\pi^*)} \rangle <u_{s,i}|u_{T,i}>$$

where $i$ is either $n$ or $\pi$ and $H_{so}$ is the spin-orbit coupling element, $u_{s,i}$ is the lowest energy vibrational mode in the $S_1$ state and $u_{T,i}$ is the vibrational mode of the $T_i$ state that overlaps with the $u_{s,i}$ mode. From symmetry arguments the spin-orbit coupling integral will only be non-zero for transitions between states with different configurations (i.e., $\Psi_{S(n,\pi^*)} \rightarrow \Psi_{T(\pi,\pi^*)}$ or $\Psi_{S(\pi,\pi^*)} \rightarrow \Psi_{T(n,\pi^*)}$). Since the steady state emission spectrum is most consistent with the excitation being $\pi-\pi^*$, the lowest energy triplet state would have to be of $n-\pi^*$ character for efficient ISC. However, both theoretical and experimental studies have shown that the lowest excited triplet state is that of the $T_1$ ($\pi-\pi^*$) regardless of the nature of the solvent. Thus, if ISC alone were responsible for the excited state deactivation the ISC would have to occur between singlet and triplet states via spin orbit coupling between the $S_1$ ($\pi-\pi^*$) and higher energy $T_n$ ($n-\pi^*$) state. In order to enhance this coupling energy the $T_n$ ($n-\pi^*$) must be lowered when 9AA is adsorbed onto the ZrP material. In addition, the vibrational overlap between the $S_1$ ($\pi-\pi^*$) and $T_n$ ($n-\pi^*$) states must also be enhanced in order for ISC to reduce the lifetime of the 9AA-ZrP material. Thus, enhanced ISC does not appear to be a viable mechanism to account for the enhanced non-radiative decay.

Non-radiate decay rates ($k_{NR}$) also occurs from the $S_1$ ($\pi-\pi^*$) state to the $S_0(\pi)$ state due to interactions with the ZrP material and this process is governed by the energy gap law:

$$k_{NR} = \langle (H_{vdw}^2)(2\pi)^{1/2}/h(\Delta E\hbar \omega_M)^{1/2} \rangle \exp(\gamma \Delta E/\hbar \omega_M)$$

(1)
\[ \gamma = \ln\left\{\frac{2\Delta E}{d\Delta M^2\hbar\omega_M}\right\} - 1 \]  

where \( H_{\text{vib}} \) is the vibronic coupling interaction energy, \( \Delta E \) is the energy difference between the excited and ground states, \( \hbar\omega_M \) is the energy of the accepting vibrational modes, \( \Delta M \) is the reduced displacement of the accepting vibrational modes and \( d \) is the number of degenerate accepting modes of frequency \( \omega_M \). Examination of Eq. 1 reveals several factors which could increase the magnitude of \( k_{\text{NR}} \) including an increase in the vibronic coupling between the excited \( S_1 \) state of 9AA (increasing \( H_{\text{vib}} \)), a decrease in the energy gap between the excited \( S_1 \) and ground \( S_0 \) states (reducing \( \Delta E \)), and/or changes in the frequency of the accepting vibrational modes (variations in \( \omega_M \)). The bathochromic shift in the emission spectrum of the 9AA-ZrP relative to 9AA in ethanol indicates a reduction in \( \Delta E \) which contributes to the increase in \( k_{\text{NR}} \). In addition, adsorption of 9AA to the ZrP material may provide a more direct vibronic coupling between the \( S_1 \) state of the 9AA and the vibrational manifold of the ZrP thus increasing the \( H_{\text{vib}} \) matrix element. This, in effect, would alter the conical intersection between the \( S_1 \) and \( S_0 \) states enhancing \( k_{\text{NR}} \) as observed in the 9AA-ZrP material.

In summary, the results presented here demonstrate the ability of 9AA to adsorb onto \( \alpha \)ZrP galleries and the association is found to produce heterogeneous populations of adsorbed 9AA with differing mechanisms of non-radiative relaxation. The excited state properties suggest reduced hydrogen bonding between the parent acridine ring and the phosphate hydroxyls.
4.4 Loading Summary:

For loading measurements, 34.5mg of dried 9AA ZrP was sonicated in exactly 1mL of ethanol for 2 minutes. To determine the number of 9AA molecules per ZrP unit 5μL of the suspension was diluted into 2mL of ethanol and was placed in a 1cm quartz cuvette and the Uv-vis absorption spectra collected. Scatter was corrected by fitting the data to a straight line and then subtracting off the resulting line (Figure 4.13).

Next the concentration, c, of 9AA in the cuvette was calculated using the corrected absorption at 402 nm and molar absorption coefficient, \( \varepsilon \), of 13 mM\(^{-1}\) cm\(^{-1}\). From this the number of 9AA molecules in the cuvette was determined using Avogadro’s number, \( N \), and the volume of solution.

\[ A = \varepsilon bc \]  

(2)
Next the amount of solid in the cuvette was converted to units of ZrP using 190g/unit mol and Avagadro’s number.

\[
\left( \frac{g}{\text{mol}} \right) \times \text{volume in cuvette} = g \text{ of solid in cuvette}
\]  

(5)

\[
g \times \frac{\text{mole units}}{190g} = \text{mole units of ZrP in solution}
\]  

(6)

\[
\text{mole units} \times N = \text{units of ZrP}
\]  

(7)

Finally the number of 9AA molecules is divided by number of ZrP units and reported as 9AA loading. The average of two samples results in a loading of 0.0022 ± 0.0009 9AA molecules/unit for αZrP mixed with 5mL of 1mm 9AA. The same procedure was followed for αZrP mixed with 5mM and 10mM 9AA ethanol solutions. The results are summarized in the table below.

\[
\frac{\text{# of 9AA molecules}}{\text{# of ZrP units}} = 9AA \text{ loading}
\]  

(8)

Theoretical loading calculations are performed first by assuming that the 9AA ring system lies parallel to the surface of ZrP. This would predict the largest surface area the molecule could occupy on the ZrP surface and the lowest possible loading. Atom to
atom distances provides the surface area of 9AA of 55.1 Å² (figure 4.15) and that one unit of ZrP is 24 Å². Therefore the theoretical amount of 9AA molecules per unit of ZrP is 0.435.

![Figure 4.15 9 amino acridine with dimensions](image)

4.5 References

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18. A. Čeklovský, A. Czimerová, M. Pentrák, J. Bujdák *J. Colloid Interface Sci.* (2008), 240-245


Chapter 5: System III- Metal-organic Polyhedra

5.1 Background

By this point, the advantage of incorporating solid state platforms with biomacromolecules to aid in understanding the effects of more physiological environments, and produce functional novel bio-materials, has been expressed numerous times. Functional materials have increased in popularity recently, as the need for novel solid state catalysis, small molecule sensors, drug delivery systems, gas storage devices has grown\textsuperscript{1-16}. These functional materials range from metal nanospheres to quantum dots, fullerenes and metal-organic frameworks.\textsuperscript{17-22} Of interest to this work, are metal-organic materials (MOMs) due to the ease at which they are made, designed and functionalized, additionally, they provide a unique ability to not only combine evolutionarily designed biological molecules to form a new bio-hybrid material, but also, the opportunity to design functional materials designed to mimic biological function.\textsuperscript{23-27} Synthesis involves the self-assembly of functionally and geometrically designed molecular building to form 2D or 3D frameworks with periodic cavities. The ability to exchange transition metals and functional organic ligands results in a diverse collection of structures and endless design options for materials with specific catalytic and physical properties.

One specific class of MOMs are nanoballs (NB), the properties (solubility, cavity size and functionality) of these discrete spherical polyhedra are easily tuned by modifying the metal or organic ligand used during self-assembly.\textsuperscript{28} Paddlewheel clusters make up the $\text{M}_2(\text{RCO}_2)_4$ building blocks where $\text{M}$ is the transition metal cation and $\text{RCO}_2$
is an organic carboxylate. The structure is described as twelve squares interconnected (the squares representing the paddlewheel clusters) at an angle of 120° by 24 benzene-1,3 dicarboxylate ligands to form a small rhombihexadron.\textsuperscript{29,30} Though a number of transition metals can form NBs, Cu\textsuperscript{2+} is the most commonly used, because it readily forms paddle-wheel complexes with organic carboxylates. The 5 position of the carboxylate ligands add another design opportunity, by varying the functional group specific properties can be tuned including size, solubility, and functionality. To date, many nanoballs have been synthesized with variation in the 5-position, dodecyloxy, sulfanato, tert-butyl and methoxy groups have all been previously synthesised.\textsuperscript{31-35} The internal cavity volumes are 0.9nm\textsuperscript{3}, with the 5- position controlling the molecular volumes, which from 9.2 to 54.4nm\textsuperscript{3}. The interior and exterior axial positions of the Cu ion coordinate to solvent molecules present during synthesis (DMSO,methanol, pyridine).

The carboxylate ligands have very specific photophysical properties which can serve as probes for environmental conditions, host-guest interactions, solvent effects and nanoball stability. This lab has previously characterized the optical proprieties of Cu hydroxyl nanoball, [Cu\textsubscript{2}(5-OH-bdc)\textsubscript{2}L\textsubscript{2}]\textsubscript{12} (CuOH NB), the nanoball of interest in this work, using both optical absorption and emission spectroscopies.\textsuperscript{36,37} CuOH NBs are formed using the 5-hydroxy substituted benzene-1,3-dicarboxylic acid and the Cu interior and exterior axial position bind either DMSO, methanol, or water. The optical spectrum of intact CuOH NB exhibits an absorption maxima at 305nm, assigned to the ligand, and a broad Cu\textsuperscript{2+} to ligand charge transfer band centered at 695nm (ε=7mM\textsuperscript{-1}cm\textsuperscript{-1}). In contrast, the free ligand and Cu\textsuperscript{2+}(NO\textsubscript{3})\textsubscript{2} in methanol adsorbs ~312nm (ε=83mM\textsuperscript{-1}cm\textsuperscript{-1}), and >750nm, respectively. The emission spectrum of the free ligand in methanol exhibits a band centered at ~360nm with a shoulder at ~390nm and is significantly
quenched when probed as part of the intact CuOH NB (Φ_{5-OH-H_2bdc} = 0.014, Φ_{OH-nanoball} = (5.6±0.5) \times 10^{-5}). It has been proposed that this quenching is a direct result of the interactions between the ligand π-system and the Cu d-orbitals, which also give rise to the 695 nm absorption band. In addition, fluorescence anisotropy measurements of the free ligand and CuOH NB in methanol showed that they are similar, even with the large differences in size. This discovery suggests the CuOH NB is intact and spherical in shape with rapid energy transfer between ligands within the structure.\textsuperscript{36}

### 5.2 CuOH Nanoball Stability\textsuperscript{1}

The majority of research available on MOPs involves synthesis and design of new structures, however information towards solution structure and stability is limited.\textsuperscript{36} Before MOPs can be utilized as platforms for drug delivery systems, and novel protein:MOP complexes a better understanding of solution properties is necessary. The work presented below utilizes the inherent optical properties of (5-OH-bdc)\textsuperscript{2-} and the intact CuOH NB to probe condition effects like temperature, pressure, water, pH and ligand exchange on CuOH Nb stability.

#### 5.2.1 Materials and Methods

All reagents were purchased from Sigma-Aldrich and used without further purification. The CuOH NB was prepared by combining equimolar amounts of Cu(NO_3)_2•2.5 H_2O and 5-OH-H_2bdc in methanol (MeOH) followed by the addition of 2 equivalents of 2,6-Lutidine or analine which is then layered over DMSO. After several days the resulting crystals were collected via centrifugation and washed several times with DMSO to insure the removal of excess reagents.

\textsuperscript{1} Portions of these results have been previously published (Vetromile et al. 2011) and are utilized with permission from the publisher.
Stock solutions of CuOH NBs were prepared by dissolving purified crystals in neat MeOH to give a final concentration between 5 and 10 mM.

In order to examine the stability of CuOH NB towards water, mixed water:MeOH solutions were prepared containing 35 \( \mu \)M CuOH NB in 2mL of solvent ranging from neat MeOH to neat water in 0.1 mole fraction increments. The stability of CuOH NB towards pH was examined using 2mL solutions of 0.2 mole fraction water:MeOH adjusted to the desired pH with HCl or NaOH. After the solution reached equilibrium CuOH NB was added from stock solutions such that the final concentration was 20 \( \mu \)M. For stability towards imidazole, 2mL of 35 \( \mu \)M CuOH NB solutions were prepared in neat MeOH. Imidazole was then titrated into the CuOH NB solution from a 50 mM stock solution (also in MeOH) to give the desired imidazole concentration. Temperature and pressure stability measurements were obtained using a Quantum Northwest variable temperature cell holder or an ISS high pressure cell, respectively. The concentration of CuOH NB was 50 \( \mu \)M for both experiments.

For water, pH, and imidazole studies UV/vis absorption spectra were recorded using a Shimadzu UV2401 spectrometer while steady-state emission spectra were recorded using an ISS PC1 (ISS, Inc., Champaign, IL) single-photon counting spectrofluorimeter. For the corresponding temperature and pressure stability measurements steady-state absorption spectra were obtained by passing a white light probe beam derived from a fiber optic cable coupled to a Xe arc lamp (Oriel) either through a 1 cm cuvette housed in a temperature controlled sample holder or through the ISS high pressure bomb and into an Ocean Optics (UV2400) CCD camera.
5.2.2 Results and Discussion

5.2.2.1 Temperature and Pressure Stability.

Figure 5.1, left displays the absorption spectra of CuOH NB in MeOH over the temperature range from 0°C to 60°C. The spectra exhibit a slight decrease in absorbance upon increasing temperature as well as a small shift in the MLCT absorption band. At high temperatures (>50°C) a green precipitate forms which may be due to the production of either isomeric structures or smaller degradation fragments (e.g., one dimensional polymers, isolated Cu-OH-bdc paddle wheel fragments, etc). Self-assembled structures have been known to contain supermolecular isomers that can form under different conditions. However, the decrease in absorbance at 695nm accounts for less than 1% loss of absorbance associated with the intact CuOH NB.

Sample concentrations are ~20 μM and the spectra were obtained in a 1-cm optical cuvette (temperature dependence measurements) or a 0.75 cm sample cell (for high pressure measurements).
Figure 5.1, right displays the corresponding absorbance changes as a function of hydrostatic pressure ranging from ambient to 3.5 kbar. The spectral changes in the charge transfer band consist of small changes in intensity and a slight hypsochromic shift. There is no evidence of significant CuOH NB degradation at pressures up to 3.5 kbar. The observed spectra changes are consistent with perturbations to the solvent.

**Figure 5.2** top left: Overlay of the 695 nm charge transfer absorption spectrum for CuOH NB with differing mole fractions of water. Top right: Corresponding overlay of the emission spectra of CuOH NB with increasing mole fraction of water. [CuOH NB] = ~20 μM. bottom Plot of absorbance changes at 695 nm and integrated emission intensity as a function of water mole fraction.
environment of the CuOH NB upon increasing pressure.

5.2.2.2 Water Stability

In order to examine the properties of MOPs in the presence of biomolecules as well as to utilize MOPs as drug delivery systems it is important to establish the effects of aqueous solvents on MOP structure and stability. Figure 5.2 top right, displays the optical spectra obtained for CuOH NB in water:MeOH solutions from 0 to 1 mole fraction water. The optical spectra in the charge transfer region show little change for water concentrations below ~ 0.7 mole fraction (90% of the nanoballs intact) while for mole fractions above ~0.7 significant loss of intensity is observed. Similar behavior is observed in the integrated emission intensity (Figure 5.2 top right ) (307 nm excitation). At low water concentrations (i.e., mole fractions below ~ 0.7) the integrated emission intensity is low due to quenching of the ligand emission by charge transfer with the coordinated Cu(II) ions. Upon increase in water concentration of ~0.7 mole fraction the integrated emission intensity increases indicating release of the coordinated 5-OH-bdc. At mole fractions above ~0.8 the integrated emission intensity matches that of the free ligand indicating complete dissociation of Cu(II).

Recent stability studies by Tonigold and Volkmer of a Cu(II) nanoball synthesized with the organic linker 5-(2-(2-hydroxyethoxy)ethoxy)benzene-1,3-dicarboxylic have also observed nanoball degradation in water/solvent mixtures. These authors compared the relative stability of the functionalized nanoball to that of the corresponding Cu paddlewheels in 1:1 V:V H$_2$O:DMF. These studies indicate that the functionalized nanoball is thermodynamically more stable than the corresponding Cu BDC paddlewheels at mole fractions of water up to ~0.7 but that the Cu nanoball degrades
over time (several months). These authors suggest that the enhanced stability of the nanoballs is due to a positive chelate affect.

5.2.2.3 pH Stability.

As the coordination of Cu(II) ions to the OH-bdc involves the deprotonated ligand the solution pH is also likely to be important for CuOH NB stability. In addition, in order to utilize CuOH NB in physiologically applications necessitates that the CuOH NB be stable under physiological pH ranges. The pH stability studies conducted here were performed in 0.2 mole fraction water:MeOH solutions to insure that the CuOH NB were not degraded due to the presence of water. The pH was adjusted using concentrated NaOH or HCl solutions and was varied from pH 3-11.5. Both steady state UV-vis and emission spectra were monitored to determine the stability of the CuOH NB over the given pH range and the results are displayed in Figure 5.3.

Figure 5.3: Panel A: Overlay of the 695 nm absorption band for CuOH NB in methanol (solid line), 0.2 mole fraction H2O methanol solutions at different pH: pH 3.0 (dashed line), pH 4.5 (dotted line), pH 8.0 (dash-dotted line), pH 10.9 (dash-dot-dot line), and H2O (short dash line). Panel B: Corresponding overlay of the emission of CuOH NB. Sample concentrations are as described for Figure 2.
At pHs below 4.5 the absorbance associated with the MLCT band at 695 nm decreases significantly, the absorbance of the parent ligand band at ~307 nm is shifted slightly to ~310 nm and the fluorescence intensity increases at 350 nm together with the appearance of a 440 nm shoulder. These results indicate some degree of degradation of the CuOH NB with an accompanying protonation of the free OH-bdc ligand. Relative to the total emission in neat water (in which the CuOH NB is completely dissociated) the intensity change at pHs below 4.5 indicate that only ~50% of the CuOH NBs remain intact. The position of the emission band at 350 nm with a weak shoulder centered at 440 nm corresponds to the protonated form, (5-OH-H₂bdc). Protonation of the free 5-OH-bdc is expected at pHs below 4.5 as the two pKₐs are 4.6 and 3.7. Previously, Tonigold and co workers examined the release of Cu²⁺ from nanoballs as a function of decreasing pKₐ. These authors found that as the pKₐ decreased degradation of the nanoball and consequential release of Cu²⁺ increased and proposed that this is a result of protonation of bound ligands prior to complete dissociation of the nanoball.

At high pH (>10) the 695 nm MLCT absorption band is completely lost and the ligand absorption band hypsochromically shifts to 300 nm from 307 nm for CuOH NB in methanol (Figure 5.4). As discussed earlier the 300 nm absorption band is assigned to the fully deprotonated form of the parent ligand. The disappearance of the 695 nm band
results from the reduction of the excited state charge transfer due to release of Cu$^{2+}$ ions from the bdc ligands. This is also consistent with an increase in emission intensity with increasing pH. The single band at 336 nm results from the fully deprotonated (5-OH-bdc)$^{2-}$ and the lack of a lower energy band indicates no inter-molecular hydrogen bonding between two ligands or ligand and solvent molecules. The fact that the ligand remains deprotonated upon CuOH NB disassembly is consistent with a mechanism through which OH$^{-}$ ions compete with the (5-OH-bdc)$^{2-}$ for coordination to the Cu$^{2+}$ ion forming the insoluble Cu(II)(OH)$_2$.

5.2.2.4 Imidazole Stability

As many biological molecules contain weakly basic...
groups the stability of the CuOH NB in the presence of imidazole has also been examined. As the concentration of imidazole increases the MLCT absorption band decreases and the emission intensity increases (figure 5.5). Again, this indicates dissociation of the structure as the imidazole coordinates to the Cu$^{2+}$. Examination of the 695 nm absorption band as a function of imidazole concentration reveals the nanoball breaks apart slowly, likely into fragments, until about 4mM imidazole where the structure is no longer intact. (figure 5.6) A Hill plot of the imidazole induced degradation of the CuOH NB reveals a $K_d$ of 2.5 mM and $n$~4. However, imidazole does not fully out-compete 5-OH-bdc for coordination to the Cu$^{2+}$ ion as the emission intensity of CuOH NB with excess imidazole does not reach that of an equivalent concentration of free 5-OH-bdc as
shown in Figure 5.7. Therefore, the addition of imidazole disrupts the spherical structure of the CuOH NB to form copper complexes containing both imidazole and 5-OH-bdc coordination. Preliminary crystallographic data (figure 5.8) suggest that at high concentrations of imidazole 2D sheets form with Cu$^{2+}$, containing linked 5-OH-bdc and axial imidazole.

The proposed mechanism was tested by crystalizing out CuOH NB in the presence of different concentrations of imidazole. Preliminary crystallographic data (figure 5.8) results showed high concentrations of imidazole formed 2D sheets with Cu$^{2+}$, containing linked 5-OH-bdc and axial imidazole.

5.2.3 Conclusions

Overall the results presented demonstrated the sensitivity of Cu based metal organic polyhedra to pressure, temperature, water, pH and imidazole. The CuOH NB are fairly robust under pressures up to ~ 4 kbar and solution temperatures up to 60°C. Both the MLCT band and the excited state emission spectra confirm the polyhedra are stable in methanol:water solutions up to a mole fraction of 0.7 (50% V:V water:methanol) but dissociate at higher water concentrations. The CuOH NB also exhibit significant degradation at solution pHs <4.5 and > 10.5 in 0.2 mole fraction water:methanol. As well as, in the presence imidazole.

The results are consistent with a mechanism of degradation in which ligands out compete the 5-OH-bdc for copper coordination ultimately resulting in the formation of Cu(2+)(L$_n$) complexes (L= 4 or 6). At low concentrations of ligand (L= water, imidazole, or OH$^-$) coordination is likely to take place at the axial positions of the Cu paddle wheel clusters weakening the Cu$^{2+}$5-OH-bdc coordination. As the concentration of L is increased the nanoball dissociates first into sheets and/or varying Cu complexes with both L and OH-bdc bound until reaching complete degradation with Cu-L$_n$ complexes in
solution (See Figure 9). In the case of imidazole, addition of higher concentrations leads to the formation of Cu complexes (layered sheets) that are not soluble in MeOH and thus complete degradation is not reached. Conversely, under acidic conditions CuOH NB degradation is facilitated by the protonation of the -5-OH-bdc ligands. Still, the fact the CuOH-NB is relatively stable in methanol:water solutions up to 50% by volume may provide a unique opportunity to probe the interactions between biological molecules and metal organic polyhedra.

5.3 Mechanism of Formation

The discovery of unique spectroscopic properties associated with intact CuOH NBs in solution not only aid in solution stability investigations, but can also be utilized to

![Figure 5.9](image.jpg)
understand the self-assembly process of these structures. To date the majority of MOP literature is focused on functionalization of current MOPs and the design of new MOP structures. Recently, studies have emerged probing the mechanism of self-assembly from solution to extended crystalline materials.\textsuperscript{42-44} For example, Rood et al employed electrospray ionization mass spectrometry to study metal carboxylate nucleation.\textsuperscript{42} These authors discovered that the assembly and crystallization of MOF[Mg\textsubscript{2}(Hcam\textsubscript{3}•3H\textsubscript{2}O)]\textsuperscript{+} SBU paddlewheels, followed by association of these paddlewheels to create cages which then assembly to form the extended frameworks. In addition, Surble et al investigated the presences of SBUs during the crystallization process of MIL-89 with the use of Extended X-Ray Absorption Fine Structure (EXAFS).\textsuperscript{43} They reported that SBUs are present in solution throughout the entire crystallization process. Though these studies do provide key insights on the assembly process, there still remains a lack of understanding on the mechanism of self-assembly in solution prior to crystallization.\textsuperscript{42-44}

More recently our laboratory reported studies examining the kinetics of CuOH NB formation in methanol using stopped flow techniques together with transient absorption spectroscopy. The 695nm charge transfer absorption band was monitored as a function of time after the rapid mixing of equimolar 5-OH-H\textsubscript{2}bdc and aniline (to deprotonate the ligand) with Cu\textsuperscript{2+}(NO\textsubscript{3})\textsubscript{2} in methanol. The data revealed two kinetic phases with lifetimes of $\tau_1=42s$ and $\tau_2=4.4s$ on time scales out to 100s. Preliminary data indicated these rates were not concentration dependent with either Cu\textsuperscript{2+}(NO\textsubscript{3})\textsubscript{2} or 5-OH-H\textsubscript{2}bdc. Interestingly, 92% of the total absorbance accumulated within the first 100ms. Further examination of the kinetics on faster time scales revealed an additional three phases, $\tau_3=310ms$ (20% of the total amplitude), $\tau_4= 26ms$ (49%), and $\tau_5<15ms$ (22%). Upon comparing these rates to the rate of Cu\textsubscript{2}(p-hydroxy benzoate)\textsubscript{4} formation (only capable of forming the
paddlewheel building blocks), it was suggested that a population of Cu\(^{2+}\)-OH-5-OHbdc clusters must form within the mixing time, <15ms.

From these results two possible Fragment Isomerization Condensation (FIC) mechanisms were proposed, one in which the isomerization/condensation process occurs simultaneously (FIC-A) and another in which this occurs sequentially (FIC-B) shown in figure 5.10. The FIC-A mechanism involves formation of large fragments consisting of varying dimensions and conformations in the first ~15ms. Specific populations of these fragments isomerize and condense with other specific fragments forming the intact nanoball. Within the FIC-B mechanism smaller fragments form during the first ~15ms which link and isomerizes forming a new population of fragments which

Figure 5.10 Scheme of the two Fragment Isomerization Condensation mechanisms
continue down this pathway with four isomerization/linkage events until the CuOH NB is fully formed.

To expand on these findings a more detailed examination was conducted to determine the effects of Cu and ligand concentrations, as well as, temperature on the kinetic rates of CuOH NB formation. The results of these studies have led to a new proposed mechanism discussed below.

5.3.1 Materials and Methods. All reagents were purchased from Sigma-Aldrich and used without further purification. The concentration dependence stopped flow experiments of CuOH NB formation were performed with varying mole ratios of 5-OHbdc:Cu$^{2+}$(NO$_3$)$_2$. The concentration of Cu$^{2+}$(NO$_3$)$_2$ was held constant at 10mg/mL in syringe 1 while 5-OHbdc with 2equivalent 2,6-lutidine was varied in syringe 2 to obtain the mole ratios 4:1, 2:1, 1:1, 1:2, 1:4. All experiments were performed in neat methanol and at room temperature.

For temperature dependence investigations kinetic traces were obtained for temperatures ranging from 5ºC to 25ºC. Syringe 1 was filled with 10mg/mL Cu$^{2+}$(NO$_3$)$_2$ and syringe 2 was filled with 3.9mg/mL 5-OH-bdc with 2 equivalent 2,6-lutidine.

A two syringe Kintek Minim-Mixer was used for stopped flow experiments. Slow time kinetics were examined using a Shimadzu UV2401 spectrometer in kinetics mode. Faster time data were collected using transient absorption instrument of our own design. The instrument consist of a 670nm diode laser (Edmond Scientific, ~3mW) as the probe source which is passed through a ¼ M single monochrometer (Yvon-Jobin) and detected using a photomultiplier tube. The signal is first amplified by a Melles Griot (13AMP005) pre-amplifier then fed through two stages of a Stanford Instruments (SR445A) 350MHz amplifier and recorded with a Tektronix TDS7404 4 Ghz DPSO. Data acquisition was
triggered by an external trigger on the stopped flow unit. Four sets of four single shot traces were acquired for each concentration and temperature. The four single shots were averaged and fit to biexponentials using Origin™. Residuals, Autocorrelation and $\chi^2$ were used to determine goodness of fit.

### 5.3.2 Results

#### 5.3.2.1 Concentration dependence

The rates of formation were examined as a function of ligand and Cu concentration to reveal the kinetic processes that are governed by diffusion. Five different mole ratios of 5-OH-5-OHbdc:Cu$^{2+}$(NO$_3$)$_2$ were examined (4:1, 2:1, 1:1, 1:2, 1:4). The concentration of Cu$^{2+}$(NO$_3$)$_2$ was held constant for each sample and ligand concentration was adjusted to obtain the appropriate mole ratios. Displayed in figure 5.11 are the fast time scale traces obtained for the four different mole ratios of ligand to copper. Each kinetic trace was fit to bi-exponentials and the results are summarized in Table 5.1.

**Figure 5.11** Concentration dependence on the fast time scales. Kinetic traces are normalized to draw focus on the differences in rates.

**Table 5.1** Summary of the concentration dependence on the rates of CuOH NB formation

<table>
<thead>
<tr>
<th>5-OH-bdc-Cu$^{2+}$(NO$_3$)$_2$</th>
<th>$T_1$ (ms)</th>
<th>$T_2$ (ms)</th>
<th>$T_3$ (s)</th>
<th>$T_4$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-1</td>
<td>13.0 ± 0.1</td>
<td>120 ± 3</td>
<td>4.2 ± 0.2</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>2-1</td>
<td>7.4 ± 0.2</td>
<td>109 ± 3</td>
<td>5.8 ± 0.3</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>1-1</td>
<td>5.4 ± 0.2</td>
<td>61 ± 2</td>
<td>4.1 ± 0.4</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>1-2</td>
<td>4.4 ± 0.2</td>
<td>47 ± 1</td>
<td>3.9 ± 0.3</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>1-4</td>
<td>1.5 ± 0.5</td>
<td>7.2 ± 0.7</td>
<td>4.3 ± 0.3</td>
<td>55 ± 3</td>
</tr>
</tbody>
</table>
The best fits give lifetimes of \( \tau_1 = 5.4 \pm 0.2 \text{ ms} \) and \( \tau_2 = 61 \pm 2 \text{ ms} \) (for 1:1 mole ratio) and these lifetimes are found to be concentration dependent.

Figure 5.12 displays the absorbance traces collected on a time scale to 200 seconds. The observed rates were found to be \( \tau_3 = 4.1 \pm 0.7 \text{ s} \) and \( \tau_4 = 58 \pm 5 \text{ s} \) and neither the rates nor the total change in amplitude exhibited concentration dependence. Interestingly, only the two early time events (\( \tau_1 \) and \( \tau_2 \)) as well as processes occurring \(< 15 \text{ ms} \) depend on copper concentration and the two final events (\( \tau_3 \) and \( \tau_4 \)) are not effected by concentration.

5.3.2.2 Temperature dependence.

To further examine if CuOH NB formation involves activation controlled steps, the kinetic rates were investigated as a function of temperature. Figure 5.13 displays the kinetic traces of four temperatures on fast time scales (each trace is the average of four data sets). The data indicates that the rates and amplitudes of \( \tau_1 \) and \( \tau_2 \) do not correspond to temperature dependent processes.
In contrast, the kinetic traces on the slower time scale, displayed in figure 5.14, reveal a significant temperature dependence. A summary of the bi-exponential fits can be found in table 5.2. The corresponding activation energies were calculated from the temperature dependence of the rates (figure 5.15). The estimated activation energy for $\tau_3$ and $\tau_4$ are $7\pm1$ kcal mol$^{-1}$ and $8\pm1$ kcal mol$^{-1}$, respectively.

Table 5.2 Summary of the temperature dependence on the rates of CuOH NB formation

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>$\tau_3$ (s)</th>
<th>$\tau_4$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>4.0±0.2</td>
<td>65±3</td>
</tr>
<tr>
<td>20</td>
<td>6.0±0.2</td>
<td>76±3</td>
</tr>
<tr>
<td>15</td>
<td>8.0±0.4</td>
<td>89±6</td>
</tr>
<tr>
<td>10</td>
<td>8±1</td>
<td>125±7</td>
</tr>
</tbody>
</table>
5.3.2.3 Computational Results

Figure 5.16  Top left Cu$^{2+}$bdc$_4$ paddlewheel all four ligands in the native positions as in the CuOH NB structure (PW$_0$) top right Cu$^{2+}$bdc$_4$ paddlewheel with one ligand rotated 180º (PW$_1$) bottom left Cu$^{2+}$bdc$_4$ paddlewheel with two ligands across from each other rotated 180º (PW$_2$) bottom right Cu$^{2+}$bdc$_4$ paddlewheel with two ligands next to each other rotated 180º (PW$_2'$) Picture provided by Christi L Whittington
Parallel to the experimental work presented here our group has been conducting computational studies to aid in determining the origins of the activation energy associated with the slow events. Previous studies of MOFs indicate that self-assembly begins with the SBU. The experimental results also establish that these building units form within the mixing time of the stopped flow instrument. For CuOH NBs the SBUs are Cu\(^{2+}\)5-OHbd\(_c_4\) paddlewheels. However, these paddle wheel assemblies may adopt multiple conformations during the assembly process. Therefore, the energetics associated with the Cu\(^{2+}\)bd\(_c_4\) paddlewheel isomerization was investigated to determine the contribution of isomerization to the total activation energy. Potential energy plots were calculated for three possible rotation mechanisms of the Cu\(^{2+}\) bound bdc ligands in the paddlewheel structure. The intact nanoball structure has all 5-OHbd\(_c\) ligands coordinated with the same orientation as displayed in figure 5.16, top left (PW\(_o\)). The variations investigated include paddlewheels with one (PW\(_1\)) or two 5-OHbd\(_c\) ligands rotated 180° (either across or adjacent, PW\(_2\) or PW\(_a\), respectively) from the nanoball orientation as shown in figure 5.16. Each of the structures were geometry optimized in the incorrect form then rotated every 15° at which point they were again energy optimized, continuing through a complete 180° rotation to the native position.

![Figure 5.17](image_url) Energy barrier of rotation of the phenyl groups in CuBDC paddlewheels, where 4_0 is the initial structure, 1_180 is one phenyl 180 degrees from initial, 2_180 is two phenyl groups 180 degrees from initial, 2-Across is 2_180 with the phenyl groups opposite each other, and 2_Side is 2_180 with the phenyl groups next to each other. Figure provided by Christi L Whittington
The first paddlewheel orientation examined was one in which a single 5-OHbdc ligand was rotated 180° from the preferred position for the intact nanoball (PW₁) shown in figure 5.16 top right and figure 5.17 black line. The potential energy results indicate the paddlewheel structure energetically favors an orientation in which one ligand is rotated 180° relative to the remaining three bound ligands. The calculated activation energy to rotate one ligand to the preferred angle for nanoball formation is 8.5 kcal mol⁻¹. The next isomerization case examined was the paddlewheel with two bound ligands adjacent to each other (as displayed in figure 5.16, bottom left) rotated 180° from the nanoballs preferred orientation (top left). The potential energy plot demonstrates that the PW₂ structure is more energetically favored than the paddlewheel with only one ligand rotated. The calculated activation energy required to rotate one while the other remains fixed is 6 kcal mol⁻¹. Rotation of the second ligand is the same as that of the PW₁ unit. The final isomerization mechanism investigated computationally involves the rotation of one ligand when in a unit in which two adjacent ligands share the same orientation that are rotated 180° from the remaining two ligands (figure 5.1, bottom right). The activation energy to rotate the first ligand is 6.5 kcal mol⁻¹ and structurally this paddlewheel orientation is not energetically favored.

5.3.3 CuOH NB Mechanism of Formation  The data presented here is consistent with a mechanism (figure 5.18) in which self-assembly of the CuOH NB begins with two fast condensation processes (τ₁=5.4 ± 0.2 ms and τ₂=61 ± 2 ms, for 1:1 mole ratio) followed by slow isomerization involving rotation of the phenyl groups of the bdc about the C-Cu²⁺ bond to the appropriate ‘native’ position (τ₃=4.1 ± 0.7 s and τ₄=58 ± 5 s) necessary for nanoball formation. Within the mixing time of the stopped flow instrument, <=15 ms, populations of Cu₂bdc₄ paddlewheels form, which is supported by the fact that Cu₂(p-
hydroxy benzoate)₄ paddlewheels display fast formation within the same mixing time. Computational investigations indicate that the fast forming population likely exists as a heterogeneous mixture of Cu paddlewheels including what has been assigned as ‘native’ paddlewheels (all 5-OHbdc coordinated with the correct angle to facilitate nanoball formation, PWₒ) as well as ‘frustrated’ structures in which one 5-OHbdc ligand (PW₁) or two 5-OHbdc ligands (either across or adjacent, PW₂ and PW₂’ respectively) coordinated at the incorrect angle, and fragments of partially ligated copper complexes (Cu₂bdc₃, Cu₂bdc₂, etc).

![Diagram of proposed mechanism for CuOH NB formation in methanol solution](image)

*Figure 5.18 Proposed mechanism for CuOH NB formation in methanol solution*
The rates of CuOH NB formation are significantly faster when the Cu concentration is in a 4 to 1 excess over ligand relative to conditions in which the ligand is in excess (see table 5.1). The population of partially formed paddlewheels in solution is largest when copper is in excess, indicating that CuOH NB formation may begin with the rapid self-assembly of small fragments of paddlewheels present in solution. This result is intuitive since the paddlewheels that constitute the intact nanoball structure share ligands and release of one or more ligands is required for the assembly of multiple paddlewheels. When ligand is in excess the majority of fragments formed within the mixing time are complete Cu$^{2+}$ paddlewheels with four ligands bound. Therefore, nucleation of small fragments to form larger fragments of the nanoball pathway is limited by the off rate of ligand from the Cu paddlewheel complex. Under excess ligand conditions this process is significantly slowed as dissociation of one ligand is likely followed by rapid coordination of another ligand free in solution.

The results suggest that coordination of small fragments formed during the dead-time of the instrument (<~15ms) condense to larger fragments in steps $\tau_1$ and $\tau_2$, followed by isomerization of the ligands within the larger fragments to completely form the CuOH NB complex. This is supported by the fact that the rates and amplitudes of final steps of formation, $\tau_3$ and $\tau_4$, are not concentration dependent but are temperature dependent. Evaluation of the rates using Arrhenius kinetics gave activation energies of 7kcal mol$^{-1}$ and 8kcal mol$^{-1}$ for $\tau_3$ and $\tau_4$, respectively. Computational investigations predict of three possible isomerization processes involving rotation of the 5-OHbdc ligands with similar activation energies suggesting these steps are isomerization events. A thermodynamic equilibrium between a large fragment containing an incorrect paddle wheel isomer and correct paddle wheel isomers in solution, (i.e. an ‘incorrect’ 5-OHbdc orientation replaced a ‘correct’) is not likely since these steps do not display
concentration dependence. Previously published work by Tonigold and coworkers found that the nanoball structure is more stable than Cu paddlewheels as result of the chelate effect. Therefore, the preference to undergo isomerization versus dissociation followed by association of an orientationally correct ligand may be due to the enhanced stability from the inter-and intra- molecular interactions of the nearly complete structure.

5.4 References:


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