HETEROGENEOUS CATALYSIS AT THE SINGLE-MOLECULE LEVEL: A QUANTITATIVE UNDERSTANDING OF THE CATALYTIC ACTIVITY OF INDIVIDUAL GOLD NANOPARTICLES AND ITS ASSOCIATED DYNAMICS

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Abstract

We use total internal reflection fluorescence (TIRF) microscopy to understand heterogeneous catalysis at the single-molecule level. This study focuses on examining, in detail, the catalytic activity of individual gold (Au) nanoparticles (5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm diameter) through single-molecule detection of a model fluorescent reaction: reduction of resazurin (non-fluorescent) to resorufin (fluorescent) in the presence of a suitable reductant (hydroxylamine). We find that for both the product formation and the product generation reaction, all Au nanoparticles follow a Langmuir-Hinshelwood mechanism. We verify the proposed mechanism for kinetic and thermodynamic consistency through temperature-dependent measurements at both the single-molecule and ensemble levels. We quantitatively study the influence of solvent on the reaction system by replacing H₂O with D₂O. Single-molecule measurements aid in deconvolution of desorption kinetic parameters, which is impossible at the ensemble-level when the desorption step is not rate-limiting. It also provides a detailed understanding of the dispersive kinetics of individual Au nanoparticles and quantifies the heterogeneity in activity among individual nanoparticles.

The next phase of our study focuses on deconvoluting the origins of temporal variations in activity present among individual catalytic turnover events on a single Au nanoparticle. We interpret the contributions of intrinsic restructuring and adsorbate-induced restructuring of gold nanoparticles by Reaction Force-Field (ReaxFF) Molecular Dynamics (MD) simulations and an autocorrelation analysis of experimental data. By adding small molecule adsorbates of varying affinity to the Au surface, we probed the contributions of the reactivity for different types of active sites on the observed variations in activity. Strong adsorbates such as thiols were found to chromatographically titrate the Au active sites from higher reactivity to lower
reactivity. By varying the coverage of thiols, we modulate the temporal variations in the activity of individual Au nanoparticles. Furthermore, at the single-molecule level, we studied the reorientation dynamics of single resorufin molecules generated on the surface of a 5.4 nm Au nanoparticle. By combining single molecule observations with theoretical calculations, we relate the presence of a slow rise in on-times (rise times) from our fluorescence turnover trajectory to the rotational dynamics of product (resorufin) on the Au surface. We perform an extensive autocorrelation analysis on the observed rise times and determined the activation barrier associated with product reorientation through temperature-dependent single-molecule analysis.

Finally, in a separate study, we probe the thermodynamic adsorption profile at a solvated organic-inorganic interface by following the binding and organization of carboxylic acid-terminated alkanethiols of varying chain lengths (C2, C3, and C6) to the surface of Au nanoparticles using isothermal titration calorimetry (ITC). The thermodynamic parameters support a mechanism of step-wise adsorption of thiols to the surface of Au NPs and secondary ordering of the thiols at the organic-inorganic interface. We observe an apparent compensation effect: the negative ΔH is compensated by a negative ΔS as the thiols self-assemble on the Au NP surface. Understanding the thermodynamics of adsorption at nanoparticle surfaces will provide critical insight into the role of ligands in directing size and shape during nanoparticle synthesis since thiols are a common ligand choice (i.e., Brust method). The ITC technique is applicable to a large number of structure-directing ligands and solvent combinations and, therefore, should become an important tool for understanding reaction mechanisms in nanostructure synthesis.
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Chapter 1

Introduction

1.1. Basic Anatomy of a Single Molecule Measurement

A classic experiment carried out at the single-molecule level investigates the properties of many individual molecules. The difficulty in directly understanding the influence of individual molecules is in the fact that even in 1 μL of a 1 μM solution, there are approximately $10^{11}$ solute molecules. The individual molecules will typically behave differently isolated from the bulk, because, in the presence of other molecules, they are dynamic, and they interact with the each other and the solvent. At the ensemble-level, a measure of the absorbance of fluorescence of the solution is a local average over all the molecules. Classical experiments have focused on determining kinetic information from these measurements over many molecules, but a great deal of information is also lost. Ensemble averages of molecular properties tend to mask the underlying reaction dynamics because the measured signals are an unsynchronized average of the contributions of every molecule in the sample. As a result, a catalytic reaction may appear to be a smooth, continuously varying process, but, in reality, each of these molecules display random and stochastic dynamics. These are associated with the fact that a typical chemical catalytic reaction generally consists of a sequence of events (adsorption, reaction, and desorption) that involve the crossing of a free-energy barrier. At the ensemble level, the combination of these individual events will allow for multiple reactions to occur and therefore can be used to determine an overall catalytic activity, which is controlled by a complex interplay of different dynamic processes. Understanding the contributions of each of these intrinsically stochastic
processes is possible, provided we study one molecule at a time. Single-molecule level measurements provide an insight into the behavior of each individual molecule and, therefore, allow the details of subpopulations in structure or dynamics within an ensemble to be deconvoluted. Additionally, single molecule measurements provide a way of probing constantly fluctuating systems under equilibrium conditions, allowing kinetic pathways to be studied without the need for synchronization. Further, single molecule measurements may allow for the observation of rare intermediates whose signal intensity is small compared to the intensity from the more abundantly populated states in an ensemble measurement.¹

There are several techniques to carry out an experiment at the single-molecule level: the two most common being optical microscopy and spectroscopy. The first, indirect detection of a single molecule in solution was pioneered by Rotman when detecting multiple reaction products of a single enzymatic molecule, β-D-Galactosidase using an optical microscope.² The important aspect of this work was to demonstrate the principal possibility of direct optical single molecule detection in a liquid, and it exercised a great influence on the further development of the field of single-molecule detection. In the recent years, single-molecule microscopic imaging technique has emerged as a powerful technique to image chemical systems. One of the important criteria for single-molecule detection is that it requires the presence of a molecule that can fluoresce in the system of interest. Fluorescence is the light emitted by an atom or molecule after a finite duration from electronically excited singlet states subsequent to the absorption of electromagnetic energy.³ Specifically, the emitted light arises from the transition of the excited species from its first excited electronic singlet level to its ground electronic level. Certain molecules absorb light at a particular wavelength and subsequently emit light of longer wavelength after a brief interval, termed
the fluorescence lifetime. The principle of fluorescence can be explained by the use of a Jablonski diagram (Figure 1.1), which shows the different photophysical processes of dye molecules. Absorbance of photons of light occurs very quickly in discrete amounts, known as quanta, and corresponds to the excitation of the molecule from the ground state ($S_0$) to an excited state ($S_1$). The energy in a quantum is expressed by the Planck’s law ($E = h\nu = hc/\lambda$; where $E$ is the energy, $h$ is the Planck’s constant, $\nu$ and $\lambda$ are the frequency and wavelength of the incoming photon, respectively, and $c$ is the speed of light). The excited molecule can relax to a lower level singlet state by the process of internal conversion and vibrational relaxation. The excited molecule returns to the ground state ($S_0$) by the emission of a photon at a longer wavelength which yields fluorescence. In some cases, the molecule can also relax through intersystem crossing into a triplet state ($T_1$). From $T_1$, the molecule can return to the ground state ($S_0$) by emission of a photon to yield phosphorescence. The three individual processes that are involved in the fluorescence emission from a molecule occur at different timescales separated by several orders of magnitude. Excitation to a higher singlet state by absorption of a photon occurs in femtoseconds ($10^{-15}$ s), the vibrational relaxation of excited state electrons to lowest energy level happens in picoseconds ($10^{-12}$ s), and emission of a longer wavelength photon and return of the molecule to the ground state (fluorescence) happens on the order of $10^{-9}$-$10^{-7}$ s. (The timescales for phosphorescence is $\sim 10^{3}$-$10^{2}$ s). The molecule capable of exhibiting fluorescence is termed as a fluorophore, which, when used for single molecule detection, should possess high photostability and high quantum yield. Quantum yield is defined as the ratio of number of photons emitted to the number of photons absorbed. Organic dye molecules are most widely used for sensitive single molecule fluorescence detection because of their high quantum yield and photostability.\textsuperscript{4}
Figure 1.1. Typical representation of a Jablonski diagram. The singlet ground, first, and second electronic states are depicted by $S_0$, $S_1$, and $S_2$, respectively. At each of these electronic levels, the fluorophores can exist in a number of vibrational energy levels, depicted by 0, 1, 2, etc. The transitions between states are depicted as vertical lines. Following light absorption, a fluorophore is usually excited to a higher energy vibrational level of $S_1$ or $S_2$ and rapidly relaxes to the lowest vibrational level of $S_1$ ($10^{-12}$ s or less). The fluorophore returns to the ground state $S_0$ from the lowest energy vibrational state of $S_1$ by emitting photons of light and the process known as fluorescence. Fluorescence lifetimes are typically on the order of $10^{-8}$ s. Molecules in the $S_1$ state can also undergo a spin conversion to the first triplet state, $T_1$. Emission from $T_1$ is phosphorescence ($10^{-2}$ s), and is shifted to longer wavelengths relative to fluorescence. Conversion of $S_1$ to $T_1$ is called intersystem crossing.
The two most common methods to perform single molecule measurements focus on diffusing fluorescent molecules and immobilized fluorescent molecules. In a single molecule diffusion experiment, a laser is focused on a small spot where optics are used to observe the fluorescence from a small sample volume ($< 0.1$ femtoliter). When the fluorescent molecule diffuses into the volume, a fluorescence photon burst is generated. The brightness, temporal persistence of the burst, and spectrum of the fluorescence contain information about the identity, size, diffusion coefficient, and concentration of each fluorophore. There is a large amount of information contained within these fluorescent bursts and techniques, such as fluorescence correlation spectroscopy (FCS), make it possible to extract the amount of time the fluctuations in the signal exist and can measure the individual properties associated with the fluorescent molecule. One disadvantage to the measurement of freely diffusing molecules is the transient nature of the observed fluorescence. To build up sufficient statistics, measurements on many different single molecules are required. The second method to carry out single molecule measurements is to immobilize the molecule of interest by tethering it to a surface (most preferably a glass slide or a glass coverslip). In such a measurement, the observation time is limited by the stability of the instrument used, the signal to noise ratio of the experiment, and irreversible photobleaching of the dye to a non-fluorescent state. One can measure the fluorescence as a function of time and can access kinetic information for single molecules. Most of the common spectroscopic and microscopic techniques capable of single molecule detection require immobilization of the molecule to a surface. The principle of operation and applications of different techniques used for single-molecule detection are detailed in Section 1.3.
Single molecule studies utilizing a fluorogenic substrate have their foundation in enzymology; enzymes were found to be dynamic entities and exhibit constant fluctuations in their catalytic rate constants.\textsuperscript{8-11} Also, most enzymatic reactions occur on the time scale of milliseconds to seconds where enzymes in an ensemble are asynchronous with each other, further complicating the study of enzymatic dynamics.\textsuperscript{12} These inherent difficulties led to single molecule level studies, which revealed the presence of heterogeneity of reactivity among individual enzymes (\textit{static variation}) and temporal variations in the reaction rate that exist for individual enzymes (\textit{dynamic variation}). One of the most relevant single molecule studies on enzymes was carried out by Lu and Xie,\textsuperscript{11} who studied turnovers of single cholesterol oxidase molecules in the enzyme’s fluorescent active site, flavin adenine dinucleotide (FAD). In their measurements, the enzyme is immobilized to a surface (glass coverslip) and using a confocal microscopy setup, an excitation laser is focused on an individual region to which the substrate solution is added. Upon conversion of the substrate by the enzyme, a highly emissive product molecule is formed that causes a burst in the recorded time-intensity trace. When this small fluorescent molecule diffuses out of the confocal volume or photobleaches, the signal decreases to its original background level. Individual turnover events are thus visualized as short bursts in fluorescence intensity versus time trace. The most crucial aspect to these types of single molecule measurements is the design of a suitable substrate capable of forming a fluorescent product molecule for the catalytic system of interest. From statistical analyses of single molecule trajectories, the authors observed significant and slow fluctuations in the rate of cholesterol oxidation by FAD. In another important study from the same group, they performed single molecule enzymatic dynamics on β-galactosidase, an enzyme which hydrolyzes the substrate, resorufin
β-galactopyranoside, yielding a fluorescent product, resorufin.\textsuperscript{13} They identified that temporal activity variations on a single enzyme has contributions from slow fluctuations in protein conformations between individual turnover events. Since proteins have many “soft” internal degrees of freedom, characterized by force constants weak enough to be significantly affected by chemical changes such as ligand binding, the notion that activity fluctuations are due to conformational changes is well accepted. The methods to study biological molecules individually have become quite advanced due to enhanced microscope capabilities and algorithms designed for the automated analysis of single molecule data.\textsuperscript{13-16} The single molecule approach offers the ultimate degree of sensitivity for detecting local structure, and dynamics of reactions. The information content afforded by a single molecule experiment markedly exceeds that offered by “bulk” ensemble measurements, yielding invaluable insights into individual molecular properties and their micro-environment.

1.2. Single Molecule Studies in Heterogeneous Catalysis

Single molecule measurements have become an extremely powerful tool for studying biological and biophysical phenomena for the past two decade, but in catalysis, the major advances have taken place in the past ten years.\textsuperscript{17-20} Fluorescence spectro(microscopy) is prevalent in the catalysis field to study enzymatic and chemocatalytic processes with high spatiotemporal resolution, a unique sensitivity down to the single molecule level and under \textit{in situ} conditions.\textsuperscript{21} Catalysis at the single molecule level can be studied by using fluorescent probe molecules and by employing a suitable spectroscopic, microscopic, or electro-catalytic technique.
The fluorescent probes designed for studying enzymatic processes can also be applied to single turnover counting on heterogeneous catalysts. This has been pioneered by Roeffaers et al. for a base-catalyzed hydrolysis-transesterification reaction on layered double hydroxide (LDH) crystals. Carboxyfluorescein diacetate (CFDA) was utilized as a fluorogenic substrate and by employing a wide-field microscope, individual turnovers were localized on hexagonal crystals. LDHs feature two distinct types of base sites: structural hydroxide groups at the basal planes, and the sites at the crystal edges due to the presence of exchanged hydroxyl anions. It was shown that in water, the hydrolysis of CFDA mainly occurred at the crystals’ edges and defect sites, while in butanol, the transesterification of CFDA occurred over the entire basal plane of the crystal (Figures 1.2 and 1.3). This study proved the existence of different types of basic sites on the different crystal facets. The same group also used the acid-catalyzed furfuryl alcohol oligomerization to monitor the catalytic activity of individual zeolite crystals in the condensed phase. With this model reaction, they observed the spatially inhomogeneous product formation inside individual ZSM-5 zeolite particles as a function of time. By following the spread of fluorescent product molecules throughout the individual crystals, they identified several crystal zones and their corresponding diffusion barriers. In a more recent study, the same group focused on the epoxidation of phenylbutadienyl-substituted boron dipyrromethene difluoride (PBD-bodipy) on micron-sized Ti-MCM-41 particles. For this system, wide field microscopy studies revealed that catalytic activity was confined to the outer regions of individual Ti-MCM-41 particles. Since reactive Ti sites were homogeneously distributed throughout the catalyst particles and sorption experiments indicated that the pore volume was fully accessible, the observations of catalytic activity were attributed to the existence of diffusion limitations.
Figure 1.2. Catalysis on individual LDH crystals. (A) Time-dependent sorption of dye molecules on an individual LDH particle. At zero time the dye solution is added on the top of the LDH crystals, resulting in a 520 nM concentration. The fluorescence intensity is represented using a false color scaling. (B) Modeling of the sorption on an LDH crystal with adsorption and desorption rate constants for zone 1, at the edge of the crystal, and for zone 2, at the basal plane. Adapted from reference [22].
Figure 1.3. Fluorescence imaging of individual LDH crystals. (A, B) Fluorescence image of an LDH crystal during transesterification of CFDA with n-butanol at (A) 40 nM and (B) 700 nM ester concentration. Every bright spot corresponds to a single catalytic reaction event at the LDH basal plane. (C) Fluorescence image of an LDH crystal during hydrolysis of 600 nM CFDA. (D) Accumulated intensity on the same crystal over 256 consecutive images, highlighting the higher contribution of the lateral faces to the total hydrolysis activity. Adapted from reference [21].
The only real-time fluorescence single molecule study on single nanoparticles has been carried out by Chen and co-workers.\textsuperscript{27-32} Using total internal reflection fluorescence (TIRF) microscopy, they examined the reduction of resazurin (non-fluorescent) to resorufin (highly fluorescent) in the presence of the reductant NH\textsubscript{2}OH over Au nanoparticles (6-13 nm diameter) with single turnover resolution. To discriminate the activity of different nanoparticles, the nanoparticles were highly diluted onto a glass surface. By observing the reaction with single turnover resolution, they could separate the adsorption and reaction events from the desorption events. When resazurin binds to a Au nanoparticle, no fluorescence was observed. Only when resazurin is catalytically converted to a resorufin molecule, a fluorescent spot is visible at the particle’s location. This fluorescent spot stays until the product desorbs from the product surface and diffuses away. When a next reactant molecule adsorsbs and is converted, the fluorescence appears again. They defined the time between two fluorescent bursts as the off-time and this represented the time needed for the reactant molecule to adsorb and to be converted to the product molecule at the Au surface. The duration of the fluorescence burst is defined as the on-time and represents the time needed for the fluorescent molecule to desorb from the Au surface (Figure 1.4A-D). Careful statistical analysis of the off- and on-times of the time-intensity trajectories, measured at different reactant concentrations, enabled them to understand the reaction mechanism. They also calculated the rate constants for the reaction and desorption process separately. They identified that the product desorbs by two distinct processes: a reactant-assisted pathway and a direct dissociation pathway. However, the structural reasons for the two different pathways could not be resolved since no direct information on particle size and morphology can be overlaid with activity measurement. The distinction between different elementary processes
is nearly impossible by traditional ensemble level measurements. Additionally, from the time-intensity plot, they observed temporal variations in single-nanoparticle catalytic activity. By determining the autocorrelation function of off- and on-times, they reported the existence of temporal activity fluctuations (dynamic restructuring) is due to nanoparticle restructuring under reaction conditions. They attributed the source of this dynamic restructuring of the nanoparticle surface to its high surface energy and continually changing adsorbate-surface interactions. Finally, the authors also identified two different types of sites on the Au nanoparticles: one with relatively stronger adsorbate binding and lower reactivity and another with weaker adsorbate binding but higher reactivity. As a function of time, the individual Au nanoparticles were able to switch between these two types of sites, due to dynamic surface restructuring.
Figure 1.4. Single-molecule nanoparticle catalysis. (A) Experimental design using total internal reflection fluorescence microscopy for studying the catalytic activity of Au nanoparticles at the single particle level. (B) A typical image (~ 18 × 18 μm2) of fluorescent products during catalysis taken at 100 ms per frame. The pixel size is ~270 nm, which results in the pixelated fluorescence spots. (C) A segment of the fluorescence trajectory from the fluorescent spot marked by the arrow in (B) at 0.05 μM resazurin and 1 mM NH2OH indicating the off and on-waiting times. (D) A segment of another fluorescence trajectory showing two on-levels at the same conditions. Adapted from reference [29].
Recently, the same group utilized super-resolution single-molecule fluorescence techniques to locate individual catalytic turnover events localized on the surface of gold nanorods\textsuperscript{33} (Figure 1.5A) of different aspect ratios as well as triangular and hexagonal Au nanoplates (Figure 1.5B).\textsuperscript{34} In both studies, the catalytic formation of resorufin was used as the model reaction in conjunction with Gaussian fitting of the point spread function (PSF) to achieve mapping of the reactive centers with a resolution of approximately 40 nm. By dissecting the catalyst particles into different subsections (end and middle sections of a nanorod or corner, edge and face sites of a triangular and hexagonal plate) (Figure 1.5C-D), the specific contribution of different types of sites to the overall reactivity was quantified (Figure 1.5E-F). For Au nanorods, which can be treated as 1D entities, it was observed that the end sites were much more reactive than the center sites and this was related to the larger fraction of under-coordinated sites present at the ends of a nanorod. For nanoplates, specific reactivity increased from the flat facet (111) regions towards edges and corners. This was attributed to the fact that for nanoplates, the highest percentage of under-coordinated sites are present in the corner regions, followed by a decreased number in the edge region, and the lowest fraction is present in the flat (111) facet region.
Figure 1.5. Site specific catalytic activity on single nanocrystals. (A, B) TEM images of a (A) Au nanorod and (B) a triangular Au nanoplate, encapsulated in mesoporous silica. (C) 2-D histogram of catalytic product locations on a single Au@mSiO2 nanorod in catalyzing the reduction of amplex red to resorufin. The red line is the structural contour of the nanorod from its SEM image. (D) Locations of catalytic products mapped onto the SEM image of a single Au@mSiO2 nanoplate. Each dot is a product molecule. The product locations are color-coded based on their regions: corners (green), edges (blue), and top facets (red). (E) Specific catalytic rate constant k of each segment of the nanorod in (C). (F) Specific catalytic turnover rate v for the three types of regions of the nanoplate in (D). Adapted from reference [31].
In the area of photocatalysis at the single-molecule level, significant progress has been made by Majima and co-workers in elucidating the formation of reactive oxygen species.\textsuperscript{35-37} They designed selective probes that specifically react with the desired reactive oxygen species. They utilized terylenediimide (TDI) and 3-(p-hydroxyphenyl) fluorescein (HPF) to selectively probe singlet oxygen and hydroxyl radicals, respectively. They immobilized these probe molecules at various distances on the surface of ETS-10 titanosilicate zeolites from the photocatalytic source of the reactive oxygen species, and measured their lifetimes and diffusion constants. Microporous titanosilicates (ETS-4 and ETS-10) are attractive materials for adsorption, ion exchange and photocatalysis because of the inherent nature of one-dimensional titania wires in the framework.\textsuperscript{38} Recently, Majima and co-workers utilized in situ fluorescence imaging of photocatalytic oxidation on single ETS-10 crystals using a fluorescent dye, 3′-(p-aminophenyl) fluorescein (APF) and were able to spatially resolve the surface active sites distributed over the ETS-10 titanosilicate zeolites.\textsuperscript{37} Their significant finding revealed that structural defects in the crystals were the preferred trapping sites for the generated reactive oxygen species. Very recently, the same group developed novel, fluorogenic probe molecules to visualize photoinduced redox reactions on single TiO\textsubscript{2} and Au-TiO\textsubscript{2} nanoparticles.\textsuperscript{38} They prepared mono-sulfonated (MS-DN-bodipy) and di-sulfonated versions (DS-DN-bodipy) of the well-known redox probe 3,4-dinitrophenyl-BODIPY (DN-bodipy) and visualized electron-transfer phenomena on the surface of the nanoparticle photocatalysts in aqueous environments (Figure 1.6A-F). Through this new fluorescent probe, they determined that Au-TiO\textsubscript{2} particles were more catalytically active than the TiO\textsubscript{2} particles for photoinduced redox reactions. Furthermore, they observed
predominant product formation in close vicinity to the 14 nm Au nanoparticles which are immobilized on the TiO$_2$ phase.
Figure 1.6. Catalysis at the single-molecule level on Au/TiO$_2$ particles. (A) Fluorescence images observed during the 488 nm laser irradiation of a single TiO$_2$ particle on the cover glass in Ar-saturated DS-DN-BODIPY (1 μM) aqueous methanol solution before and after UV irradiation. (B) Typical fluorescence intensity trajectory observed for a single 14 nm Au/TiO$_2$ particle in an Ar-saturated DS-DN-BODIPY (50 nM) aqueous methanol solution under 488 nm laser irradiation. The fluorescence bursts are highlighted in red. The gray
dashed line indicates the threshold level separating the on and off states. (C) Fluorescence intensity distribution corresponding to a single product molecule on a 14 nm Au/TiO₂ particle. To determine the centroid position, a 2-D Gaussian function was fitted to each image. (D, E) Spatial distributions of fluorescence spots (red dots, > 50 spots) collected from (D) TiO₂ and (E) 14 nm Au/TiO₂ particles. The SEM images of the particles analyzed are shown. Scale bars are 100 nm. The locations of the reactive sites and Au nanoparticle are surrounded with dashed lines in red and blue, respectively. Adapted from reference [37].
Mulvaney and co-workers have recently studied the reaction rates of a redox reaction involving the oxidation of ascorbic acid by dissolved oxygen on a single Au nanocrystal surface using surface plasmon spectroscopy. The direct electron transfer reaction is extremely slow in an aqueous solution, while in the presence of a Au catalyst, electrons are injected into the nanoparticle by the ascorbic acid. They identified electron transfer rates of ~4600 electrons per second during the oxidation of ascorbic acid and determined chemical reaction rates that correspond to 65 O$_2$ molecules per second during oxygen reduction. This study provides the first direct measurements of both oxidation and reduction steps of a redox reaction on a single nanocrystal.

1.3. Single Molecule Techniques to Understand Heterogeneous Catalysis and Photocatalysis in Liquids

Optical microscopy and spectroscopy have grown into two of the most powerful techniques for exploring the individual nanoscale behavior of molecules in complex local environments. This section reviews the experimental single molecule microscopic and spectroscopic techniques in heterogeneous catalysis and photocatalysis with emphasis on studies in liquids.

1.3.1. Total Internal Reflection Fluorescence (TIRF) Microscopy

Since its discovery in 1984 by Daniel Axelrod, TIRF microscopy has served as a high sensitivity (signal-to-noise ratio) technique that can be used to obtain a very thin optical section of a specimen whilst minimizing background noise and can be used to observe single molecules in liquid-phase. The principle of TIRF is based on the exponential decay of an evanescent wave generated upon total internal reflection of a light wave when it passes from
a high refractive index (glass or quartz coverslip) to a low refractive index medium (air or water) (Figure 1.7). Total internal reflection (TIR) is only possible in situations in which the propagating light encounters a boundary to a medium of lower refractive index. Its refractive behavior is governed by Snell’s law:

\[ n_1 \times \sin \theta_1 = n_2 \times \sin \theta_2 \]

where \( n_1 \) is the higher refractive index and \( n_2 \) is the lower refractive index.

The angle of the incident beam, with respect to the normal to the interface, is represented by \( \theta_1 \), while the refracted beam angle with the lower-index medium is given by \( \theta_2 \). When light strikes the interface at a sufficiently high angle, termed the critical angle (\( \theta_c \)), its refraction direction becomes parallel to the interface (90° relative to the normal). At \( \theta_c \), refraction occurs at 90° (\( \sin \theta_2 = 1 \)), and Snell’s law reduces to:

\[ n_1 \times \sin \theta_c = n_2 \]

\[ \theta_c = \sin^{-1} \left( \frac{n_2}{n_1} \right) \]

When the laser illumination is adjusted in such a way that the incident angle (\( \theta_i \)) is greater than the critical angle (\( \theta_c \)), the illuminating beam is entirely reflected back into the microscope slide upon encountering the interface, and an evanescent wave is generated in the lower refractive index medium immediately adjacent to the interface, in a direction normal to the solid-liquid interface. For glass water interface, the value of \( \theta_c \) is 62.3°.
Figure 1.7. Schematic illustration of the basic concept of total internal reflection fluorescence (TIRF). The molecules (blue in figure) are supported on a glass microscope slide. The refractive indices of glass and aqueous medium are compatible with TIR within the glass slide. When the illuminating laser beam is incident at an angle greater than the critical angle, it is entirely reflected back into the glass slide upon striking the solid-liquid interface, and an evanescent wavefront is generated in the aqueous medium immediately adjacent to the interface. Any fluorophore present adjacent to the glass surface (within 100 nm) are excited (red) by interaction with the evanescent field and can be detected.
The range over which excitation is possible is determined by the evanescent wave intensity \( I \) in a direction perpendicular to the interface, which drops off with distance \( z \) into the low refractive index medium and is given by,

\[
I(z) = I(0)e^{(-\frac{z}{d})}
\]

where, \( I(0) \) is the intensity at the interface, and the exponential decay distance \( (d) \) is expressed as,

\[
d = \frac{\lambda_0}{2\pi} (n_2^2 \sin^2 \theta - n_1^2)^{-1/2}
\]

where, \( \lambda_0 \) is the wavelength of incident illumination, and \( n_1, n_2 \) represent the refractive indices of low (air/water) and high (glass/quartz), respectively.\(^{20}\) For green laser (\( \lambda = 532 \text{ nm} \)) illumination, the values of \( d \) range from 100-150 nm at \( \theta_i \gtrsim \theta_c \), and this indicates that excitation of the photons extends to only a short distance from the interface into the low refractive index medium. This corresponds to the primary advantage of TIRF as a technique, the fluorophores that are sufficiently close to the solid-liquid interface are only excited, while those present further away from the interface are not, and this leads to minimal interference from the background. Another advantage of TIRF is the low absolute powers of the laser that are required to illuminate the samples.

There are two basic approaches to configuring a system for TIRF: the prism method and the objective lens method (Figure 1.8A-B).\(^{20}\) In the prism technique, a focused laser beam is introduced into the microscope coverslip through a prism attached to its surface, and the beam incidence angle is adjusted to the critical angle (Figure 1.8A). Reliance on a prism for introduction of the laser beam has several limitations, primarily due to geometric
constraints on the sample of interest, i.e., with the prism placed on the top of the glass microscope slide, it becomes difficult to move the coverslip and the microscope slide and also to inject reactant solution into the space between coverslip and microscope slide. Another disadvantage of the prism technique is that in most configurations based on inverted microscope designs, the laser illumination is introduced on the sample side opposite to the objective optics, requiring imaging of the evanescent field region through the bulk of the specimen. If the prism is placed on the objective side, this presents additional complexity due to the close proximity of a short working distance between the objective to the sample and the prism position. Even though the prism configuration has been used in biological applications for over two decades, it has never materialized into a mainstream TIRF setup technique.
Figure 1.8. TIRF illumination configurations through (A) prism method and (B) objective lens method. (A) In the prism technique, a focused laser beam is introduced into the microscope coverslip by means of a prism attached to its surface. (B) In the objective lens method, an high-NA objective is employed to introduce the laser beam to the coverslip-sample interface. Figure reproduced from the Nikon TIRF website. (http://www.microscopyu.com/articles/fluorescence/tirf/tirfintro.html)
A different configuration based on coupling the input laser beam through the periphery of a high-NA (NA – numerical aperture) microscope objective lens is currently widely used for most TIRF applications. The input laser beam must be focused at the extreme edge of the objective rear aperture to ensure that light will exit the front optical surface at an angle equal to or greater than the critical value (Figure 1.8B). This method was first implemented for single molecule detection by Funatsu et al. The main advantages of this set-up is that by confining illumination at the rear focal plane of the objective, light rays from the center of the illumination cone that would normally emerge at sub-critical angles are blocked. The resulting emission from the objective is a hollow cone of light incident upon the TIR interface at an angle sufficient to create total internal reflection. If significant illumination is passed through the central portion of the objective rear aperture, this would result in epi-illumination rather than total internal reflection. This lowers the signal-to-noise ratio and one can easily switch between epi-illumination and TIRF by simple adjusting a mirror placed in front of the laser input path so that it can either be coupled to the edge of the objective or through the center. TIRF has been extensively used in biological studies at the single molecule level; it has only been recently applied to single molecule heterogeneous catalysis. The primary limitations of TIRF are that it requires immobilization of the catalyst on the glass/quartz coverslip and also the fact that one of the molecules involved in the reaction needs to be fluorescent.

The first application of TIRF to study metal catalysis was performed by Chen and co-workers. They studied the chemical catalysis of gold nanoparticles of various sizes at the single-particle level and the electrocatalysis by single-walled carbon nanotubes (SWNTs), both at single-turnover resolution. By utilizing the conversion of resazurin (non-fluorescent)
to resorufin (fluorescent) on gold nanoparticles as a model reaction, they quantified particle-dependent and time-dependent activity fluctuations on different-sized gold nanoparticles and attributed it to dynamic reconstruction of the nanoparticle surface.\textsuperscript{29,31} The reaction solution was flowed over the Au nanoparticles immobilized on to the surface of a coverslip and the formation of each fluorescent product molecule was followed under laser illumination at TIRF conditions. This provided a real-time single-reaction resolution detection of single nanoparticle catalysis. For the electrocatalysis experiments, they dispersed individual SWNTs onto a conductive ITO surface which served as the working electrode, and was coupled with a Ag/AgCl reference electrode and a Pt counter electrode. The non-fluorescent resazurin was flowed over the working electrode and was converted into the fluorescent resorufin and observed by TIRF illumination in real-time.\textsuperscript{42} By utilizing a point spread function (PSF) analysis, individual active sites on the SWNTs were identified. In a more recent study by the same group, it was demonstrated that one can quantify dynamic structure-activity relationships at the single molecule level on gold nanorods and nanoplates.\textsuperscript{33,34,43} They utilized TIRF to quantify the catalysis of the nanorods at a temporal resolution of a single catalytic reaction at a spatial resolution of $\sim 40$ nm yielding information about the reactivity differences within a single nanorod and among individual nanorods.\textsuperscript{33} These studies have been geared towards understanding a specific chemistry occurring on the catalyst surface by utilizing TIRF as a single molecule level detection technique. In the near future, due to its high sensitivity and reduced background noise, TIRF can be used to understand a number of catalytically controlled reactions at the single molecule level, wherein the product molecule is fluorescent in nature and remains unobscured by ensemble averaging.
1.3.2. Confocal Microscopy

The advent of single molecule fluorescence detection has revolutionized fluorescence microscopy. As the quality of light sources improved, even the extent of emission from individual fluorophores could be distinguished from the background. Several optical scanning configurations have been demonstrated to serve the basic requirements for single molecule detection and one such microscopic technique that is widely used is confocal microscopy. In confocal microscopy, a diffraction-limited laser is utilized as the point source (Figure 1.9). The excitation laser is focused to a nearly diffraction limited volume and the light collected from the sample passes a spatial filter to reject background contributions arising from outside the excitation volume. The diameter of the laser focus can be defined by several criteria: the Rayleigh criterion and the Sparrow criterion are the most common ones.
Figure 1.9. Confocal fluorescence microscopy setup. The collimated excitation light from the source is reflected by the dichroic mirror onto the objective lens. By overfilling the back aperture the light is then focused into a small spot. The fluorescence light is collected by the same objective lens and is transmitted by the dichroic mirror.
In both criterion, the background scattering scales with the illumination area. This can be overcome by employing a high NA objective, which improves the signal-to-noise by both increasing the signal collected and by reducing the background scatter. The emitted fluorescence light and backscattered light are collected by the objective and the residual laser light is filtered out. Before reaching the detector, fluorescent light is focused by a microscope tube lens through a pinhole aperture located at the microscope image plane. This pinhole serves to reject out-of-focal plane light and its diameter determines the absolute depth of a confocal image. A smaller pinhole provides superior axial resolution at the expense of transmission, and is difficult to align and a suitable size of the pinhole is essential for processes where background fluorescence needs to be minimized. The fluorescence intensity can be measured by a point detector (avalanche photodetector, APD; or a photomultiplier tube, PMT) which efficiently detects photons with low-dark current (a PMT will produce a small current even without incident photons and this is referred to as the dark current), resulting in good signal-to-noise ratio. These measurements can be used to monitor fluorescence lifetimes with a microsecond temporal resolution. Emission spectra can also be measured with a CCD camera combined with a polychromator. The major advantages of confocal microscopy over conventional optical microscopy are (i) it is possible to obtain higher resolution as the resolution increases with shorter wavelengths and is higher as the NA of the objective increases, (ii) better contrast can be achieved as all undesirable light is neglected, (iii) it is possible to obtain optical slices of different planes by changing the pinhole aperture and the focal plane, (iv) details such as fluorescence intensity, decay time, and emission spectra can be collected, and (v) with the slices obtained in the different focal
planes and due to its small femtoliter excitation volume, it has been demonstrated that one can utilize confocal microscopy to study single molecules in three dimensions.24

Confocal microscopy is prevalent in the field of single-molecule liquid-phase heterogeneous catalysis and Rooffaers et al. have introduced this technique to follow catalytic chemistry in zeolites.24,25 The liquid-phase acid-catalyzed oligomerization of furfuryl alcohol in dioxane was used as a reporter reaction for imaging the catalytic activity of coffin-shaped ZSM-5 crystals. Using confocal microscopy, they have demonstrated the existence of internal diffusion barriers and located in 3D, the emissive reaction products formed within the zeolite crystals. Through this reaction, they observed the spatially inhomogeneous conjugate oligomer formation as a function of time. These studies along with others have focused on gaining detailed insights into the intergrowth structures of zeolite crystals.46-48 In another contribution by the same group, they have followed the acid-catalyzed formation of a stable fluorescent diarylcarbenium (carbocation) from the dehydration of 1,3-diphenyl-1,3-propanediol inside the zeolite structure.12 By utilizing confocal microscopy and 14 different styrene derivatives, the authors showed that the fluorescent carbocation formation rate under experimental conditions is mainly determined by the diffusivity and the stabilization of the carbocation. Confocal microscopy can also be applied to materials research, where the primary focus is on understanding the dynamics of polymer molecules. With single molecule detection, confocal microscopy can be applied to investigate the various processes that occurs at the glass transition.49 Confocal fluorescence microscopy has emerged as a potentially useful in situ characterization technique for heterogeneous catalysis in both liquid and gas-phase. Due to its high spatiotemporal resolution and sensitivity, it is possible to probe, localize, and study kinetics at the single-
molecule level on a catalyst particle under working conditions, to follow inter- and intra-particle transport, and to determine the release of organic molecules from inorganic hosts.\textsuperscript{45}

1.3.3. \textit{Fluorescence Correlation Spectroscopy (FCS)}

FCS is an experimental technique developed to study kinetic processes in liquid phase through measurement of fluorescence intensity fluctuations.\textsuperscript{7} FCS is based on the analysis of time-dependent intensity fluctuations that are a result of some dynamic process, typically translation diffusion into and out of a small volume (voxel) defined by a focused laser beam and a confocal aperture. The fluctuations in intensity can also be a result of rotational diffusion and other photodynamic process which can be used to identify the formation of intermediates and transient states.\textsuperscript{50} The dimension of the focused laser beam, together with the confocal pinhole of the microscope, defines the observation volume from which fluorescence is collected (Figure 1.10).
Figure 1.10. (A, B) Experimental set-up for an FCS instrument.51 (A) A laser beam, corrected for polarization is focused by a high-NA objective lens (OBJ) onto a fluorescent sample (S). The epifluorescence is collected by the same objective, reflected by a dichroic mirror (DM), focused by a tube lens (TL), and is focused onto a detector (DET) after passing through a filter (F). (B) Magnified focal volume (green) within which the sample particles (black circles) are illuminated by the laser light. (C) A typical fluorescence signal, as a function of time, measured for rhodamine green (RG) with a λ of 488 nm.
The basis of FCS is the autocorrelation function of the detected fluorescence intensity fluctuations. The temporal evolution of concentration fluctuations can be quantified by the corresponding fluctuations in the fluorescence signal around its mean value. The signal can be correlated with itself at a later time to obtain the autocorrelation function $G(\tau)$. The autocorrelation function of fluctuations in fluorescence emission carries information on the characteristic time scales pertaining to each individual process and the relative weights of transition in the system of interest (Figure 1.11). In a typical FCS measurement, there is more than one molecule in the observation volume simultaneously. The behavior of different independent molecules is completely uncorrelated and results in a constant background in the autocorrelation function. Only the contribution from the one molecule results in a characteristic time, and can be identified from the time-dependent section in the autocorrelation function.
Figure 1.11. A representative FCS curve of Green fluorescent protein (GFP) in aqueous solution highlighting the timescales associated with the presence of rotational and translation diffusion, and fluorescent fluctuations related to transient intermediates. Adapted from reference [16].
FCS is the oldest technique to be used in the field of single molecule fluorescence spectroscopy. The initial limitations were due to high background light levels and quantum yields that were too low for sufficient detection, which required extremely long measurement times and high concentrations of fluorescent molecules. Recent developments including the introduction of extremely small detection volumes in combination with confocal epi-illumination, highly sensitive CCDs and APDs for fluorescence detection, and very selective bandpass filters to separate fluorescence from the background, have improved the signal-to-noise ratios in FCS measurements by several orders of magnitude, and has positioned FCS as a promising technique towards single molecule detection.\textsuperscript{6} The main advantage of FCS is that the allowance for continuous observations of single molecules towards measurement of diffusion and reaction kinetics even for systems in equilibrium provided the reversible process caused spectral changes. In addition, FCS measurements do not require surface immobilization like other spectroscopic techniques and can be performed in solution. FCS can primarily be employed for molecules that have diffusion coefficient in the range of $10^{-5}$-\textasciitilde$10^{-11}$ cm\textsuperscript{2}/s. The limitation of this technique is mainly concerned with data interpretation as it involves correlation functions and complex statistical and diffusive equations.\textsuperscript{53} Typical applications of FCS include evaluation of fast diffusion processes in the field of single molecule diffusion and chemical kinetics, protein association reactions, and DNA hybridization.\textsuperscript{7,54} For example, in inorganic-organic chemistry, excitation light is focused into a tight, diffraction limited spot inside an inorganic host particle. Any fluorescent organic dye molecule diffusing through this small volume will generate a fluorescence signal. Several such single molecular events can be collected and by computing its temporal autocorrelation functions, one can determine the diffusion coefficient and guest-host interactions between
organic molecules and the inorganic porous hosts.\textsuperscript{55} FCS thus provides a convenient method to determine dynamics of single molecules in solution. Depending on the length and time scale of interest, the fluorescent molecule and its concentration, FCS can be considered a technique with single molecule detection resolution.

1.3.4. \textit{Surface-Enhanced Raman Scattering (SERS) Spectroscopy}

SERS is a Raman Spectroscopic technique that provides enhanced Raman signal from Raman-active compounds that have been adsorbed onto metal surfaces. These metal surfaces are called SERS-active substrates and can be classified into metallic nanoparticles in colloidal solutions (the most common being colloidal gold and silver particles in the 10-100 nm size range), planar metallic structures, such as arrays of metallic nanoparticles supported on a planar substrate (glass, silicon, or metals) and metallic electrodes.\textsuperscript{56} The main advantage of SERS over Raman Scattering is that the former is both surface selective and highly sensitive; increases in the intensity of the Raman signal in the order of $10^4$-$10^8$ have been observed.\textsuperscript{57} SERS selectivity of surface signal results from the presence of surface enhancement mechanisms present only at the surface. There are two primary mechanisms of enhancement for SERS: an electromagnetic and a chemical enhancement. The electromagnetic effect is based on the interaction of light with the nanostructured metal surface, which excites an oscillation of conduction electrons known as the localized surface plasmon resonance (LSPR). This resonance is dependent on the presence of the metal surface roughness features. Excitation of the LSPR results in amplification of both the incident and scattered fields at the metal surface, leading to very large enhancements in Raman scattering ($10^4$-$10^8$). The chemical mechanism is perceived to be an enhancement in polarizability caused by direct interaction between the adsorbed molecule and the metal surface. Chemical
enhancement involves changes to the adsorbate electronic states due its chemisorption to the surface. However, the contribution to overall enhancement from chemical mechanism is low ($10^{-10}$) in comparison to electromagnetic effects.$^{58,59}$ The sensitivity of SERS can be utilized for single molecule detection and in situ adsorbate-surface analysis of electrochemical, catalytic, biological, and organic systems.$^{60,61}$ Surface modification techniques extend the applicability of SERS beyond coinage metal surfaces to many nanomaterials of catalytic interest. There has been a growing interest to focus on transition metals such as Pt, Pd, Ru, Fe, Co, and Ni for SERS, a deviation from the typically probed Au, Ag, and Cu surfaces.$^{62}$ In situ characterization of the adsorption and reaction of molecules at catalytic interfaces is extremely important for the ultimate goal of rational catalyst design.

Fluorescence measurements has been one of the most widely used methods for single-molecule detection. However, it is limited by the fact that one of the molecules need to be labeled for fluorescence detection.$^{20}$ The emergence of single-molecule SERS (SMSERS), which yields more chemical information, can potentially overcome these limitations due to its important advantages over fluorescence measurements. The most attracting aspect of SMSERS is its high specificity providing a unique fingerprint of the target molecule under study. This allows for easier detection of the SMSERS signal from any background signals. SMSERS can be directly applied to any molecule without labeling, whereas fluorescence requires the presence of a chromophore. SMSERS can be carried out at principally any excitation wavelength (depending on the substrate), while fluorescence in most cases, is typically limited to the visible range. In addition, SMSERS does not have any problems associated with photobleaching and photodecomposition, which is a well-known drawback.
associated with fluorescence. SMSERS was pioneered by Nie and Emory when they detected Raman scattering from single Rhodamine 6G molecules adsorbed on Ag nanoparticles at extremely dilute concentrations (< 10^{-10} M).\textsuperscript{63} A second study by Kneipp et al. focused on SMSERS of crystal violet on Ag nanoparticles in solution where fluctuations in signal intensity and statistical analysis were used as evidence of single-molecule behavior.\textsuperscript{64} A recent review by Le Ru et al. and a perspective by Lee et al. have shown the possibility of SMSERS towards developing tailored substrates with reproducible single molecule activity, in particular, SERS enhancement factors on the order of 10^{12}-10^{14} can be achieved corresponding to an effective SERS cross section of about 10^{-16} cm^2/molecule thereby allowing SERS detection of single molecules.\textsuperscript{58,65} From an experimental standpoint, it is vital that we mention the difficulties/limitations associated with SMSERS detection. The two main limitations deal with the ideal location of the molecule in order to be detected and the observation, analysis and interpretation of the observed SMSERS signals. However, recent progress has been made towards addressing these limitations, which may prove to be vital towards the existence of SMSERS as an effective single-molecule detection technique.\textsuperscript{66}

SERS provides vibrational informational over a wide spectral range and is more attuned with measurements at liquid/solid interfaces due to its high structural sensitivity and compatibility with water, an inherent advantage when compared to other surface techniques like Sum Frequency Generation (SFG) spectroscopy and Attenuated Total Internal Reflectance Infrared (ATR-IR) spectroscopy. A recent study by Heck et al. demonstrated the ability of SERS to study the catalytic hydrodechlorination of 1,1-dichloroethane by Pd islands grown on Au nanoshells in water at room temperature.\textsuperscript{67} They utilized Au nanoshells to enhance the catalytic activity of Pd islands and provide sufficient surface enhancement and
observed a number of intermediates in the reaction sequence in real-time. This coupled with another study at elevated temperatures by Stair and coworkers reveals the potential of SERS to observe surface adsorbates in a liquid environment and thereby understand catalysis at the liquid/solid interface.\textsuperscript{68}

1.3.5. Surface Plasmon Resonance (SPR) Spectroscopy

SPR spectroscopy is a powerful label-free detection technique to monitor noncovalent molecular interactions in real-time. Typical information that can be obtained from an SPR experiment include details about specificity of an interaction at the surface, kinetic (binding affinity, dissociation and association rate constants) and thermodynamic parameters (enthalpy, entropy, and activation energy) of selected molecules present in the sample of interest.\textsuperscript{69} The detection principle relies on an electric charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under conditions of total internal reflection (TIR). When incident light propagates from a medium of higher refractive index to a medium of lower refractive index, and at an angle greater than the critical angle ($\theta_c$), the incident light is completely reflected and TIR occurs. At the point of reflection at the interface, an evanescent wave will penetrate into the medium of lower refractive index to a depth in the order of $\frac{1}{4}$ of the incident wavelength.\textsuperscript{70} If a semitransparent noble metal film is placed at the interface, then in conditions of TIR, SPR can occur. This is commonly known as the Kretschmann configuration (Figure 1.12).\textsuperscript{70,71} In SPR, the evanescent wave excites electrons present within the metal layer of a metal-dielectric interface, yielding surface plasmons. In the Kretschmann configuration, a prism coupler is used for the optical excitation of surface plasmons. The incident wave vector is given by the following expression:
where $K_i$ is a component of the incident wave vector parallel to the prism interface, $\theta$ is the incident light angle, $\lambda$ is the wavelength of incident light and $n_p$ is the refractive index of the prism. The wave vector of the plasmon mode is represented by:

$$K_p = \frac{2\pi}{\lambda} \sqrt{\frac{\epsilon_m \epsilon_d}{\epsilon_m + \epsilon_d}}$$

where $K_p$ is the surface plasmon wave vector and $\epsilon_m$ and $\epsilon_d$ are the dielectric permittivity constants of the metal film and the dielectric medium, respectively. SPR occurs when $K_i = K_p$. 
Figure 1.12. Schematic representation of coupling of light to a surface plasmon through Kretschmann configuration.70 A light wave passes through a high refractive index ($n_p$) prism and is totally internally reflected at the base of the prism, generating an evanescent wave penetrating into a thin metal film. The evanescent wave propagates along the interface with propagation constant ($K_i$), which can be adjusted to match that of the surface plasmon ($K_p$) by controlling the angle of incidence ($\theta$).
Typical applications of SPR include heterogeneous catalysis, interfacial chemistry, self-assembled monolayers, RNA/DNA sequencing, and protein folding. One of the first applications of SPR in single molecule heterogeneous catalysis was studied by Novo et al. who observed the steady state accumulation of redox reactions catalyzed by single nanocrystals. They directly observed the kinetics, electron injection and extraction during a redox reaction involving the oxidation of ascorbic acid on a single gold nanocrystal. They measured electron transfer rates of ~ 4600 electrons per second and chemical reaction rates involving 65 molecules of O₂ per second. Another recent study by Zijlstra et al. focused on binding of single protein molecules to the biotin receptors on the surface of a gold nanorod and was detected by monitoring the longitudinal SPR at a single frequency using photothermal microscopy. The major limitations to single-molecule sensitivity are the intrinsic SP band shift per electron added and the signal-to-noise efficiency of the CCD spectrometer used for current measurements. In the near future, improved signal-to-noise ratios open up a realm of opportunities for single molecule studies, where one can monitor the functioning of a particular catalyst at single electron transfer rates.

This section has described in detail the important spectroscopic and microscopic techniques that are in current use to understand heterogeneous catalysis at the single molecule level under ambient conditions. There are several other non-fluorescence based techniques that have gained prominence in the field of single molecule detection. One such technique that requires mentioning is scanning probe microscopy (SPM) since it has arguably the highest spatial resolution for imaging single molecules. SPM can be divided into two categories – Atomic Force Microscopy (AFM) and Scanning Tunneling Microscopy (STM). AFM uses a cantilever with a sharp tip at its end to raster scan the surface to
construct the morphology image of the sample. The quality of AFM imaging is often related to the sample preparation, tip radius, tip contamination, and the operator’s experience.\textsuperscript{75} AFM has nanometer spatial resolution (about 50 nm) and can be used for single-molecule detection. AFM can be combined with chemical force microscopy (CFM) to allow for chemical specificity. However, the major limitation to application of AFM to study catalytic processes at the single-molecule level is due to its poor time resolution. It takes several minutes to obtain one image with conventional AFM, which is much longer than the timescales of many catalytic processes.\textsuperscript{75} STM relies on tunneling current between a tip and a sample, and a conductive substrate is required. Tunneling electrons from a STM can be used to excite and induce changes in a molecule. STM is suited for investigation of nanoscale chemical and physical phenomena with an emphasis on probing the basic properties of single atoms and molecules on solid surfaces. One of the main drawbacks of STM has been its lack of chemical sensitivity. Ho and co-workers have demonstrated that chemical analysis with STM is possible with inelastic electron tunneling spectroscopy (IETS) and have reached the limit of sensitivity of vibrational spectroscopy, that of a single bond. They have measured spatially resolved vibrational intensity with sub-angstrom resolution in single molecules.\textsuperscript{76} They have separately studied the formation of a metal-carbonyl bond\textsuperscript{77}, determined and characterized the intermediates in the formation of CO$_2$ by the catalytic oxidation of CO at metal surfaces\textsuperscript{78}, and hydrogen transfer reaction with single-molecule detection using STM-IETS.\textsuperscript{79} However, until recently, real-time, solution condition measurements were considered technically challenging by using STM. Hulsken et al. imaged single molecules of manganese porphyrin catalysts at a liquid-solid interface using STM.\textsuperscript{80} They monitored oxidation
catalysis and determined that oxygen atoms from an O\textsubscript{2} molecule are bound to adjacent porphyrin catalysts on the surface before their incorporation into an alkene substrate.

In all forms of fluorescence microscopy and spectroscopy, the ultimate aim is to extract the maximum amount of information from each photon generated. In addition, the above described techniques can be coupled with other detection methods such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), and this could offer a plethora of opportunities to understand catalysis at the nanoscale using single molecule fluorescence spectroscopy and microscopy.

1.4. Prospects of Single Molecule Methods Towards Aspects of Catalyst Design

Our world faces a variety of challenges in creating alternative fuels, reducing toxic waste in manufacturing, preventing future pollution, and creating safe pharmaceuticals. Catalysts are needed to meet these challenges but their complexity and diversity will require a revolution in the way catalysts are designed and used. Nanoparticles are the most common type of heterogeneous catalysts and are widely used as catalysts in countless industrial processes. There have been substantial advancements in the ability to control shape and composition during nanoparticle synthesis, opening the door for catalysts highly tuned to their specific applications. Opportunities to understand and predict how these catalysts work at the atomic scale and nanoscale are now appearing; made possible by breakthroughs in the past two decades in powerful predictive methods, including density functional theory (DFT), and novel measurement techniques for imaging catalysts and reactants.\textsuperscript{22,81,82} The fundamental insight into catalytic processes gained from single-molecule measurements should enable more efficient catalyst design by providing a dynamic, rather than static
picture of the influence of catalyst structure on activity and selectivity. In doing so, it will be possible to selectively activate a single bond in a multifunctional reactant to produce a desired product. It will also enable a predictive capability in catalyst science and allow for the design of new catalysts and catalytic processes that approach the ultimate goal: highly active, stable catalysts that provide near 100% selectivity to a desired product with minimal use of energy.

Catalysts increase the rates of reactions that proceed via sequences of steps such as diffusion, adsorption, reaction, and desorption. The temporal behavior of these steps, along with transport phenomena, contribute significantly the overall rate of the reaction. Designing new catalysts requires detailed information concerning these individual steps and, in particular, information on which are rate-limiting so that a catalyst can be structured to facilitate these steps without a negative impact on other elementary operations. Also, the dynamic processes of catalysis involve motion and energy transfer on length scales ranging the atoms and electrons of the substrate-catalyst combination to that of large-scale reactors. The processes span huge ranges of time scales, from femtoseconds at the quantum level to years in catalyst deactivation. Integration of these dynamic processes provides a rigorous foundation for prediction of kinetics of catalytic processes. Single-molecule imaging and spectroscopic methods can be used for characterizing dynamic events with superior time and space resolution, both on catalysts and single nanostructures. The motions and rearrangements of the substrate and the catalyst atoms can be detected and specific locations of catalytic turnover events can be determined. The observation of distinct types of catalytic sites and location of the most active sites on a catalyst was a major breakthrough established by single molecule techniques. However, in order to design the most efficient catalyst,
one needs to understand which structural differences within the catalyst are responsible for its catalytic activity. This requires mapping the catalyst structure during turnover conditions and overlaying this structural map simultaneously with the activity map of the catalyst. This can be achieved by single molecule microscopy/spectroscopy techniques in combination with high resolution electron microscopy techniques. Advances towards such a combination have already started and the work by Chen and co-workers, who have combined single molecule microscopy (TIRF) with scanning electron microscopy (SEM) in order to precisely identify the locations of catalytic turnovers on a single catalyst particle and reveal the presence of reactivity gradients, has provided insights into single molecule methods which will influence catalyst design.\textsuperscript{34}

Current knowledge of catalyst design leading to the facile formation of desired products with high activity/selectivity and minimal environmental impact is still quite rudimentary. Chemical conversion is sustainable if the catalysts effectively control chemical conversion with perfect atom efficiency and minimize energy utilization; preferably by taking advantage of alternate energy sources (solar and/or electrochemical). Catalysts in nanoparticulate form represent the predominant catalytic entity in most energy conversion processes. They either directly promote the chemical conversion or facilitate chemical transport to active interfaces. Catalysts for the production of solar fuels couple photo absorption into catalytic cycles involving oxidized carbon. With limited final product targets, hybrid processes using chemical catalysts will further convert solar fuel to advanced chemicals. Single molecule methods offer a unique perspective in understanding the activity of these photocatalysts through time and space resolved observation of fluorescence products generated during a photocatalytic reaction and related them to the catalyst structure. Recent
success of Majima and co-workers on elucidating the most active sites on a photocatalyst (Au embedded on TiO$_2$), through single molecule microscopy, have revealed the applicability of these techniques to reveal the mechanism of heterogeneous chemical reactions on semiconductor nanoparticles and to characterize the structure of different photocatalysts, thereby leading to improvements in their design.$^{35-38}$

Finally, a major effort of research in the field of catalyst design has been dedicated to the development of single-site heterogeneous catalysts that can efficiently tailor the catalyst properties.$^{85}$ In single-site heterogeneous catalysts, active sites are well-defined, evenly distributed, and have distinct chemical surroundings. These single sites are typically located on solid supports with high surface area and consist of a limited number of atomic species which are spatially isolated from each other, structurally well characterized, and have identical energies of interaction between the site itself and a reactant. Understanding the activity of these single-site systems using single molecule techniques, in real-time, can allow for the design of ideal catalysts for desired reactions rather than adapt existing catalysts for a particular reaction by employing a conventional trial-and-error approach.

1.5. Summary of the Dissertation

This section briefly describes the contents in each of the chapters of this dissertation in order. Chapter 1, the present chapter, elaborates on the need for studying catalysis at the single-molecule level and provides an account of the advances in heterogeneous catalysis at the single-molecule level. This introductory chapter highlights the different single molecule microscopic and spectroscopic experimental techniques currently applied to study heterogeneous catalysis, leading up to the prospects for single molecule approaches with
efforts focused on improving the efficiency of an existing catalyst and designing new ones. Chapter 2 contains the single-molecule level kinetic analysis of catalytic activity of individual Au nanoparticles by focusing on the reduction of non-fluorescent resazurin to fluorescent resorufin, as the model reaction, through the use of total internal reflection fluorescence (TIRF) microscopy. A reaction mechanism is proposed and verified for its kinetic and thermodynamic analysis through temperature-dependent single-molecule measurements. Additionally, this chapter highlights the variations in activity among individual catalytic turnover events on a single nanoparticle, which demonstrates the highly dispersive nature of individual Au nanoparticles. Chapter 3 presents a single-molecule level study to understand the origins of the variation in activity prevalent with single Au nanoparticles. The same model reaction presented in Chapter 2 is utilized and carboxylic-acid terminated thiols of varying alkyl chain length are used as effective active sites reductants for Au nanoparticles. The intrinsic reactivity of different types of active sites is related to the observed variations in activity between individual turnovers. A method to effectively modulate these variations in activity is also discussed. Chapter 4 presents a unique study on the observations made at the single-molecule level related to the reorientation dynamics of the fluorescent product molecule upon formation on the Au surface. This single-molecule analysis complements the results obtained from theoretical predictions of rotational dynamics and also highlights the presence of an activation barrier for rotation. Chapter 5 presents the thermodynamic adsorption profile at a solvated organic-inorganic interface which is probed by following the binding and organization of carboxylic acid-terminated alkanethiols of varying chain lengths (C2, C3, and C6) to the surface of gold nanoparticles (NPs) using isothermal titration calorimetry (ITC). The effects of alkyl chain length,
temperature, and Au NP size on the energetics at an organic-inorganic interface are discussed. Chapter 6 draws the appropriate conclusions from the preceding chapters and establishes the basis of future work in obtaining new aspects of catalytic design (active site information) by carrying out single molecule measurements on microplates. Furthermore, a new model catalytic reaction to understand structure-selectivity realtionships at the single-molecule level is discussed. The basic types of catalytic chemistry that possibly be carried out by utilizing small molecule fluorescent probe molecules are discussed. A detailed account on the different classes of fluorescent probe molecules that exist and the potential single-molecule reactions that can be used to understand different catalytic chemistry at the single-molecule level is also presented.

1.6. References


Chapter 2

Exploring Solvent Inhibitory Effects and Dispersive Nature of Catalytic Gold Nanoparticles: A Single-Molecule and Ensemble-Level Kinetic Analysis

2.1. Introduction

Heterogeneous catalysts play an important role in the manufacture of chemical intermediates for the petrochemical, pharmaceutical, and specialty chemical industries due to the presence of various active sites that lower the activation barrier for different reactions. The activity of a catalyst is dictated by the surface properties of these active sites and the ability to probe reaction kinetics on a catalyst surface is essential for understanding various surface processes, including adsorption, reaction, and desorption. The elementary steps within catalytic cycles can be characterized by a separation of time scales during catalysis and the kinetic effects, which are dispersive in nature, can be studied with the time scale of each step. The dispersion in chemical kinetics can be accompanied by changes in kinetic parameters, such as activation energy, adsorption enthalpy, and desorption energy; the latter two are applicable in a heterogeneous catalytic reaction. In addition, in a liquid-phase reaction, the presence of solvent is effective in aiding in desorption to free active sites and to dissipate possible reaction exothermicity. While there are numerous benefits to heterogeneous catalysis with a liquid phase solvent, there are potential issues that make it difficult to deconvolute the interactions occurring on the surface and their impact on the activity. A primary issue arises from the fact that the solvent is in massive excess, which makes it difficult to study the influence on kinetics. Overall, the solvent can act as a potential
participant in the overall reaction kinetics and liquid-phase kinetic studies have focused primarily on activity and selectivity of the overall process; however, the understanding of the true nature of solvent effects in the reaction system is still rudimentary. \(^{12-14}\)

Reaction kinetics involving fluorescent molecules presents a novel method to understand chemical kinetics and focuses on the variation in the temporal duration of emissive and non-emissive periods (i.e., off-on lengths) during a chemical reaction. \(^{15}\) Single-molecule measurements provide an effective means to understand dispersive kinetics prevalent in catalytic reactions in both homogeneous and heterogeneous systems. \(^{3,4,16-20}\) They have shed light on the heterogeneity in reactivity among individual particles (static variation) and shown that temporal fluctuations in the reaction rate exist for single particles (dynamic variation). \(^{21}\) However, unlike enzymes, an individual NP has a number of active sites that behave differently but are clearly indistinguishable from one another with the spatial resolution available. \(^{22}\) When considered on a particle basis, dynamic variation and static variation cannot be differentiated from one another. NP kinetics with single turnover resolution for the reduction of resazurin to resorufin using hydroxylamine (NH\(_2\)OH) as the reductant over Au NPs have been examined by Chen and co-workers using a single molecule fluorescence approach. \(^{14,20,23-26}\) They proposed the kinetics of resorufin formation follows a Langmuir-Hinshelwood mechanism, while resorufin dissociation (desorption) from the NP surface occurred via two different pathways: a resazurin-assisted dissociation pathway (at low concentrations of resazurin, < 1 μM) and a direct dissociation pathway (at saturating concentrations of resazurin). \(^{20}\) Their results were based on observing individual catalytic turnovers on Au NPs at room temperature and lacks the rigorous testing necessary to validate a proposed reaction mechanism for kinetic and thermodynamic consistency according to
classical heterogeneously-catalyzed kinetics. The proposed mechanism based on single-molecule measurements was not verified at the ensemble-level as the catalytic kinetics was deemed to be complex due to particle-particle interactions. This is important as all industrial catalytic reactions are carried out at the ensemble-level. Furthermore, the experimental conditions need to be carefully manipulated in order to allow single-molecule observation. Therefore, when one discusses single-molecule data, it should definitely be cross-examined by other experimental techniques, most preferably, the same system investigated at the ensemble-level.

Single-molecule level measurements provide dynamic insight into the behavior of each individual molecule and allows for the subpopulations in a structure or the dynamics within an ensemble to be delineated. In addition, single-molecule measurements also provide a way to understand kinetic pathways without the need for synchronization. Ultimately, the catalytic kinetics at both the single-molecule and ensemble level must satisfy the same reaction mechanism. Using a single-molecule fluorescence approach, we study liquid-phase catalysis of individual Au nanoparticles for the reduction of resazurin (non-fluorescent) to resorufin (fluorescent) in the presence of a reductant (hydroxylamine). We find that the reaction kinetics at both single-molecule and ensemble level can be adequately described by a unifying Langmuir-Hinshelwood mechanism invoking two distinct types of sites are necessary for the reaction. We aim to understand the influence of the solvent and report the effect of competitive adsorption between the solvent and the reactants on the observed kinetic parameters when H2O is replaced with D2O. The measurement of single NP kinetics as a function of temperature enables the determination of changes in kinetic and thermodynamic parameters necessary to support the proposed reaction mechanism. The
utility of these single molecule measurements, studied as a function of temperature, is apparent because they enable simultaneous determination of $E_a$ and $E_{des}$ through Arrhenius plots in a single measurement. It is extremely difficult to deconvolute this much information from ensemble measurements since all information related to the desorption energy is lost, unless this step is rate-limiting. Finally, we simultaneously deconvolute $E_a$ and $E_{des}$ from single nanoparticle studies as a function of temperature, quantify their dispersive nature, and comment on the kinetic and thermodynamic validity of the proposed reaction mechanism.

2.2. Experimental

2.2.1. Materials

Resazurin, resorufin, hydroxylamine (NH$_2$OH), and deuterium oxide (D$_2$O) were obtained from Sigma Aldrich (Milwaukee, WI). Hydroxylamine d$_3$-DCl was obtained from CDN isotopes (Quebec, Canada). Citrate-protected Au NPs (5 nm) in aqueous dispersions were obtained from Ted Pella (Redding, CA). All chemicals were used as received. Milli-Q grade water (18.2 MΩ·cm) was used in all experiments.

2.2.2. Temperature-Dependent Single-Molecule Experiments

Single molecule experiments were performed on an Olympus IX-71 total internal reflection fluorescence (TIRF) microscope. The microscope is illuminated using a continuous wave (CW) Argon/Krypton-ion laser (35-KAP-431-220, Melles Griot, Carlsbad, California, USA) with 476 to 676 nm excitation; output power 4 to 20 mW. A CW vertically-polarized 520 nm beam was focused on the sample for the fluorescence excitation of resorufin. An oil immersion objective (PlanAPO 60×/1.45 NA, TIRFM-2, WD 0.15 mm) was used for collection of the fluorescent photons. For TIRF, laser light is coupled via a fiber-optic cable
to the TIRF illuminator on one end of the epiport and focused off-center on the back aperture of the objective. A Peltier-cooled 12-bit digital CCD camera (SensicamQE) with high resolution, sensitivity, and high quantum efficiency (65%) in the visible range was used to detect photons emitted by resorufin. The camera was modified with a fast shutter to enable the collection of two successive images with an interval of 500 ns. The camera was operated at 45 msec time resolution for all the single molecule experiments. Movies of fluorescence bursts were analyzed using a home-written Mathematica program (Appendix A), which extracts the individual fluorescence intensity trajectories from localized fluorescent spots over the entire duration of the movie.

A 40 mm glass cover slip is functionalized with 3-aminopropyltriethoxysilane (APTES) and citrate-protected 5.4 nm Au NPs are electrostatically-adsorbed onto this surface. The cover slip is incorporated into a perfusable fluid optical cavity which is fixed on the stage of the microscope (Figure 2.1). The top surface of the microaqueduct slide is coated with an electrically conductive transparent film of Indium-Tin oxide (ITO) and is used to remove heat from the optical cavity. Temperature control is provided by circulating a coolant fluid through an O-ring sealed window cooling adapter placed on the surface of the microaqueduct slide. The Au nanoparticles are separated from the cooling fluid and are in contact only with the reactant solution. The temperature is measured by placing a T-Type thermocouple (Omega Engineering Inc., Stamford, CT) on the surface of the microaqueduct slide.
Figure 2.1. (A) Schematic representation of the flow chamber arrangement for temperature-controlled single molecule experiments. The cooling water flows through the FCS2 cooling adapter. Taken from www.bioptechs.com/Products/FCS2. (B) Photograph of the flow chamber with cooling adapter. A mixture of resazurin and NH$_2$OH is pumped through ports located on the sides of the optical cavity, where it directly interacts with the Au nanoparticles. The flow characteristics of the reactant solution, while in the optical cavity, are controlled by selecting gaskets of specific geometry (14 × 24 mm, rectangle – A1424, Bioptechs Inc., Butler, PA) that direct the flow. A cooling water circulator pump is used to create a flow of fluid through the heat exchange cavity (the space in between the microaqueduct slide and the glass window of the cooling adapter) in the optical cavity.
2.2.3. Temperature-Dependent Ensemble-Level Measurements

We carried out ensemble-level kinetic measurements to determine the activation energy for the reduction of resazurin to resorufin in excess NH$_2$OH catalyzed by 5.4 nm Au NPs. The aqueous catalytic reactions were carried out using a Shimadzu UV-3600 UV-Vis-NIR spectrophotometer at different temperatures (20 - 45°C) and were initiated by adding 100 μL aliquots of aqueous 5 nm Au nanoparticle solution into a premixed solution of resazurin and NH$_2$OH. Temperature control was provided by S-1700 Shimadzu temperature control unit connected to a thermoelectric cell holder. The quartz cuvettes containing the reactant sample and a reference quartz cuvette (milli-Q water) are placed inside the dual-cell holder. Dry-air was purged through the cell holder to prevent condensate formation on the cell surface when conducting low temperature measurements. Upon injection of Au NPs, the solution gradually turns from blue (resazurin) to red (resorufin). Resazurin absorbs at 600 nm and resorufin absorbs at 570 nm. The instrument is set-up to record the absorbance at both 600 and 570 nm with a time resolution of 100 msec.

A control experiment demonstrated Au NPs were required for the reduction of resazurin by NH$_2$OH. Additionally, the reductant, NH$_2$OH was required for the reduction of resazurin over Au NPs. To convert the absorbance data into concentration, we determined extinction coefficients of both resazurin and resorufin. The extinction coefficient of resazurin at 600 nm was determined to be $\varepsilon_{600\text{ nm}} = 32537$ M$^{-1}$ cm$^{-1}$ and resorufin at 570 nm was $\varepsilon_{570\text{ nm}} = 54242$ M$^{-1}$ cm$^{-1}$.

2.3. Results and Discussion

2.3.1. Detection of individual catalytic turnover events at the single-molecule level
To systematically probe the effects of temperature and solvents on catalytic kinetics and understand their influence on dispersion of kinetic parameters, we tracked individual catalytic turnover events on single 5.4 nm Au NPs (Figure 2.2) by monitoring the real-time reduction of resazurin to resorufin by NH$_2$OH. By maintaining a constant flow of resazurin (0.05 - 8 μM) and NH$_2$OH (1 mM), we measured individual fluorescent bursts due to the production of a single molecule of resorufin on the surface of a 5.4 nm Au NP, by interfacing the temperature controlled cell with a TIRF microscope (Figure 2.3A). The individual turnovers are represented by photon bursts of arbitrary duration and “dark” periods, also of arbitrary length (Figure 2.3B). The “dark” period, also referred to as the waiting time between bursts ($\tau_{off}$), represents the time taken for the reactant molecules to diffuse, adsorb on the Au surface and react to form the product molecule. Once product is formed, the waiting time ($\tau_{on}$) represents the desorption behavior (direct measure of surface residence time) of resorufin, which can be recorded as the length of the fluorescent burst. The individual turnover trajectories were collected on the same 5.4 nm Au NP at four different temperatures (Figure 2.3C). Addition of excess citrate to the solution (up to 100 mM) had no effect on the observed activity, i.e., the calculated turnover frequency (TOF), $<$TOF$>$ = ($<$\tau_{off}$>$ + $<$\tau_{on}$>$)$^{-1}$ (where $<$ denotes average values) was found to be constant (Figure 2.4). Similar conclusions were reached by Xu et al.$^{20}$ The observed activity fluctuations are not caused by the citrate used to synthesize the Au NPs and the surface coverage of citrate is not a determining factor in observed activity fluctuations.
Figure 2.2. (A) TEM Micrographs of Au NPs used in our study and (B) the corresponding particle size distribution. From the particle size distribution (347 Au NPs), the average diameter of the Au NPs is $5.4 \pm 0.7$ nm.
Figure 2.3. (A) Experimental schematic of a flow reactor used to image temperature dependent catalytic turnover events on the surface of individual Au NPs. A perfusable optical cavity is used to control the temperature. A round glass coverslip (40 mm diameter) was initially covered by alkylamine groups to which citrate-protected 5.4 ± 0.7 nm Au NPs were electrostatically-bound. The reactant solution is passed over the Au NPs by means of polyethylene tubing connected to the top slide at a flow rate of 5 μL/min. The non-fluorescent resazurin is converted to the highly fluorescent resorufin when illuminated by a 520 nm laser. (B) A segment of a fluorescent trajectory of a single 5.4 nm Au NP for the conversion of resazurin to resorufin in the presence of NH$_2$OH at single turnover resolution at 25 °C. Embedded image of the bright spot represents the formation of a product molecule (on) and the blank image shows its desorption (off). (C) A segment of the fluorescence turnover trajectory of a single 5.4 nm Au NP at four different temperatures.
Figure 2.4. Values of $<\tau_{\text{off}}>^{-1}$ and $<\tau_{\text{on}}>^{-1}$ in the presence of excess sodium citrate. The experiments were carried out with 4 μM resazurin and 1 mM NH$_2$OH at 25 °C. Error bars are S.D.
2.3.2. Reaction kinetics for resorufin formation and desorption at the single-molecule level and the ensemble level

At the single-molecule level, the individual waiting times are dispersive (stochastic) in nature and their average values $<\tau_{\text{off}}>$ and $<\tau_{\text{on}}>$ represent the time-dependent single-particle rates of resorufin formation and resorufin desorption, respectively. For the reduction of resazurin to resorufin, when averaged over individual turnover events from multiple Au nanoparticle, both $<\tau_{\text{off}}>$ and $<\tau_{\text{on}}>$ result in saturation kinetics with increasing concentration of resazurin while NH$_2$OH is in large excess (Figures 2.5A and 2.5B). The dependence of $<\tau_{\text{off}}>$ on resazurin concentration is expected, but the dependence of $<\tau_{\text{on}}>$ on resazurin concentration indicates that resazurin influences the product dissociation from Au NP surface. At the ensemble-level, at a fixed NH$_2$OH (1 mM) and Au NP (83 nM) concentration, the initial reaction rate shows an increase with increasing resazurin concentration and eventually saturates at high resazurin concentration (Figure 2.6A). Similarly, at a fixed resazurin (4 μM) and Au NP (83 nM) concentration, NH$_2$OH also demonstrates saturation kinetics (Figure 2.6B). In addition, at constant resazurin (4 μM) and NH$_2$OH (1 mM) concentration, the initial reaction rate followed a second order dependence on Au NP concentration (Figure 2.6C); the slope of the plot between ln (initial rate) vs ln (Au NP concentration) resulted in a value of 2 (inset, Figure 2.6C). This indicates that the reactants (resazurin and NH$_2$OH) bind non-competitively to two distinct types of sites of the surface of Au NP. Provided the adsorption of reactants were competitive, we should have observed a first order dependence of the initial rate on Au NP concentration indicating the presence of only one type of surface sites.$^{30}$
Interestingly, at all temperatures, the observed rate from single-molecule and ensemble-level measurements indicate that the reaction rate at the ensemble-level is an order of magnitude smaller than the reaction rate at the single-molecule level (Figure 2.5 and 2.6). This magnitude of this difference in rates is significant and we demonstrate this depends on the percentage of active Au nanoparticles at the ensemble level. In addition, Figure 2.5A and 2.6A demonstrates a lag in reactivity between the experiment at the single molecule and the ensemble level, i.e., the rate versus resazurin concentration plot for ensemble measurement requires a higher resazurin concentration compared to the single molecule measurement. We demonstrate that this lag in reactivity is due to influence on competitive adsorption between solvent and resazurin for the same set of active sites on the active Au surface at the ensemble-level. Although the binding between solvent and resazurin remains competitive at the single-molecule level, but due to the biased nature of the single-molecule measurements, we observe a reaction event only when solvent has desorbed, allowing resazurin to bind to the surface and react. The measurement of higher rates at the single-molecule level most likely suggests that a reaction event occurs in the absence of influence of solvent. It is important to note that the single-molecule and the ensemble-level measurements are essentially two different modes of studying the same reaction and therefore should be described by a unifying reaction mechanism.

Based on the observed kinetic results at the single-molecule and the ensemble level, the reduction of resazurin to resorufin can be described by Langmuir-Hinshelwood formulation with the following sequence of elementary steps. Resazurin and NH$_2$OH adsorption was considered to occur molecularly in a non-competitive manner on two different types of sites, and quasi-equilibrium between non-adsorbed and adsorbed species
was assumed. Water was assumed to compete for the same set of active sites as the reactants and maintain quasi-equilibrium.

\[
\begin{align*}
A + S_1 & \rightleftharpoons AS_1 & (1) \\
W + S_1 & \rightleftharpoons WS_1 & (2) \\
B + S_2 & \rightleftharpoons BS_2 & (3) \\
W + S_2 & \rightleftharpoons WS_2 & (4)
\end{align*}
\]

\[
AS_1 + BS_2 \xrightarrow{k_r} R_{on}S_1 + X S_2
\]

\[
R_{on}S_1 \xrightarrow{k_d} R_{off} + S_1
\]

where, \(A = \) Resazurin, \(S_1 = \) Au site to which resazurin binds, \(W = \) Water, \(B = \) NH\(_2\)OH, \(S_2 = \) Au site to which NH\(_2\)OH binds, \(R = \) Resorufin, \(X = \) oxidation products of NH\(_2\)OH. \(R_{on}S_1\) refers to the product molecule resorufin in the “on” state still bound to the surface of the Au NP. \(R_{off}\) refers to the product molecule in solution outside of the TIRF detection zone. \(K_A\) and \(K_B\) are equilibrium constants associated with adsorption of resazurin and NH\(_2\)OH on the Au NP surface. \(K_{W1}\) and \(K_{W2}\) are the adsorption equilibrium constants for water on two different surface sites \(S_1\) and \(S_2\). The rate constants, \(k_r\) and \(k_d\) are associated with formation and desorption of resorufin, respectively.
2.3.2.1. Reaction Kinetics at the Single-Molecule Level

At the single-molecule level, the formation of resorufin follows a Langmuir-Hinshelwood mechanism which accounts for the reactant adsorption and reaction by maintaining fast adsorption equilibrium for the reactants (resazurin and NH$_2$OH). From the elementary steps described, the reaction step to distinctly represent formation of resorufin is:

$$AS_1 + BS_2 \xrightarrow{k_{r,SM}} R_{on}S_1 + XS_2$$  \hspace{1cm} (7)

Note that Eq. 5 is similar to Eq. 7, but $k_r$ is replaced by the $k_{r,SM}$, the single-molecule rate constant of the reaction. If $k$ represents the catalytic rate constant for one active site and $n_1$ and $n_2$ represents the number of resazurin and NH$_2$OH molecules bound to the Au surface, the rate of formation for one molecule of resorufin is $kn_1n_2T$. According to the principles of Langmuir isotherm$^{30}$,

$$n_1n_2 = n_{1T}n_{2T}\theta_A\theta_B = n_{1T}n_{2T} \frac{K_AC_{A0}}{1 + K_AC_{A0}} \frac{K_BC_{B0}}{1 + K_BC_{B0}}$$  \hspace{1cm} (8)

where $\theta_A$ and $\theta_B$ represent the fractional coverage of resazurin and NH$_2$OH, $n_{1T}n_{2T}$ represents the total number of catalytically active sites on a Au NP. $C_{A0}$ and $C_{B0}$ represent the concentration of resazurin and NH$_2$OH, respectively. At any given point in time, the concentration of resazurin and NH$_2$OH will correspond to their initial concentration. This is due to the fact that we observe the reaction at single-turnover resolution, i.e., formation of one product molecule. By virtue of a single-molecule kinetic analysis, we obtain the Langmuir-Hinshelwood expression to determine $<\tau_{off}>^{-1}$ (see section 2.6.1 for complete derivation).

$$<\tau_{off}>^{-1} = \int_{0}^{\infty} \frac{1}{\tau_{off}(\tau)}d\tau = kn_{1T}n_{2T}\theta_A\theta_B = k_{r,SM} \frac{K_AC_{A0}}{1 + K_AC_{A0}} \frac{K_BC_{B0}}{1 + K_BC_{B0}}$$  \hspace{1cm} (9)
where \( f_{\text{off}}(\tau) \) is the probability density function of \( \tau_{\text{off}} \), and \( k_{r,SM} = kn_{1T}n_{2T} \) takes into account the combined activity of all catalytically active sites on an individual Au NP. Eq. (9) predicts \( <\tau_{\text{off}}>^{-1} \) follows a hyperbolic dependence with increasing resazurin concentration and saturates towards \( k_{r,SM} \) at high resazurin concentrations. The concentration of \( \text{NH}_2\text{OH} \) is in excess and hence, in Eq. 9, the term \( (K_B C_{B0}/1+K_B C_{B0}) \approx 1 \). Fitting Eq. 9 to the observed kinetic data by a weighted least-squares method in Figure 2.5A results in the value of \( k_{r,SM} \) and \( K_A \) (Table 2.1). The values of \( k_{r,SM} = 0.31 \pm 0.04 \text{ s}^{-1} \) and \( K_A = (6.9 \pm 1.2) \times 10^6 \text{ M}^{-1}\) obtained from our experimental analysis at 25 °C on 5.4 nm Au NPs are consistent with the results previously published by Zhou et al.\(^{25}\) on individual 6 nm Au NPs.

Once the product, resorufin, forms on the Au surface, it desorbs and is convectively transported out of the TIRF zone. The reaction sequence that represents resorufin desorption is given by,

\[
R_{\text{on}} S_1 \xrightarrow{k_d} R_{\text{off}} + S_1
\]

\[\text{AS}_1' + BS_2 \xrightarrow{k_{r,SM}} R_{\text{on}} S_1' + XS_2'\]  \(\text{Eq. (10)}\)

Here \( S_1' \) and \( S_2' \) represent two different sites on which a second molecule of resorufin may be formed. This reaction sequence for determining \( <\tau_{\text{on}}>^{-1} \) is distinctly different from the one derived by Xu et al.\(^{20}\) as they identified that the product dissociates either through binding of a second resazurin molecule or by spontaneous dissociation. Eq. (10) presents a scenario wherein a second product molecule is generated at a different site on the same Au NP immediately following the desorption of the first product molecule from the NP surface. This reaction sequence happens within the time resolution of the experiment, resulting in an extended \( \tau_{\text{on}} \), and should be included in determining the single-molecule rate of product
desorption. The presence of Eq. 10 can account for the resazurin concentration dependence on the rate of product desorption represented in Figure 2.5B. The equation connecting $<\tau_{on}>^{-1}$ with the described kinetic parameters (see section 2.6.1 for complete derivation) is:

$$\langle \tau_{on} \rangle^{-1} = \frac{1}{\int_{0}^{\infty} \tau f_{on}(\tau) d\tau} = \frac{k_{com}(1 + k_{d})^2}{k_{d}(k_{d} + 2k_{com} - 1)} \quad (11)$$

where,

$$k_{com} = k_{r,SM} \frac{K_{A}C_{A0}}{1 + K_{A}C_{A0}} \frac{K_{B}C_{B0}}{1 + K_{B}C_{B0}} \quad (12)$$

where $f_{on}(\tau)$ is the probability density function of $\tau_{on}$. Eq. (11) predicts $<\tau_{on}>^{-1}$ follows a hyperbolic dependence with increasing resazurin concentration and at saturating concentrations reduces to,

$$\langle \tau_{on} \rangle^{-1} = \frac{k_{r,SM}(1 + k_{d})^2}{k_{d}(k_{d} + 2k_{r,SM} - 1)} \quad (13)$$

The value of the desorption rate constant ($k_{d}$) can be determined by substituting the value for reaction rate constant ($k_{r,SM}$) in Eq. (13) (Table 2.1). The ability to determine $k_{d}$ represents an advantage of performing kinetic measurements at the single-molecule level. It is important to note that all kinetic parameters determined from fitting Eq. (9) and (11) are NP-averaged results to yield a single NP basis. Eq. (9) and (11) predicts $<\tau_{off}>^{-1}$ and $<\tau_{on}>^{-1}$ will show distinct differences in saturation levels and initial slopes, based on different values of $k_{r,SM}$, $K_{A}$, and $k_{d}$ intrinsic to individual Au NPs (Figure 2.7). This further highlights the heterogeneity in nanoparticle reactivity and highly dispersive kinetics prevalent between individual Au NPs. In particular, the study by Xu et al.\textsuperscript{20} presented three different types of behavior for $<\tau_{on}>^{-1}$; increase, decrease, or constant value of $<\tau_{on}>^{-1}$ with increasing resazurin
concentration for individual Au NPs which supports their proposed reaction mechanism. Our study indicates an increase in $<\tau_{on}>^{-1}$ with resazurin concentration (Figure 2.7B), but with different saturation levels for each individual Au NP.
2.3.2.2. Reaction Kinetics at the Ensemble-Level

The mechanism proposed at the single-molecule level should also hold true at the ensemble-level. At the ensemble-level, we assume formation of resorufin \( (R_{on}) \) to be an irreversible rate determining step (Eq. 5). The differences in rate obtained from the kinetic study suggest that the presence of water leads to a possible inhibitory effect, which leads to the inclusion of water species in the site balance along with molecularly adsorbed resazurin and \( \text{NH}_2\text{OH} \) species. We observe saturation kinetics with \( \text{NH}_2\text{OH} \) concentration in our ensemble-level measurements and believe that \( \text{NH}_2\text{OH} \) should be involved in the rate-determining step of the reaction. Our observations are consistent with a more recent observation by Alejo et al.\(^{31}\) at the ensemble-level. They identified that the reaction rate for resazurin reduction depends on \( \text{NH}_2\text{OH} \) concentration and therefore, should be incorporated in the rate-determining step of the reaction. The rate of disappearance of resazurin is a function of concentration of resazurin, \( \text{NH}_2\text{OH} \), and Au nanoparticles. Based on a Langmuir-Hinshelwood formulation (see section 2.6.1 for complete derivation), the rate for resazurin disappearance is given by,

\[
-r_A(C_A, C_B, C_{NP}) = k_{r,ens} n_{1T} n_{2T} N_{eff} \frac{K_AC_A}{1 + K_W C_W + K_A C_A} \frac{K_BC_B}{1 + K_W C_W + K_B C_B} C_{NP}^2
\]

(14)

where \( C_A, C_B, C_W \ (= 55 \text{ M}) \) and \( C_{NP} \ (= 83 \text{ nM}) \) represent the concentration of resazurin, \( \text{NH}_2\text{OH} \), water and Au NPs, respectively. \( k_{r,ens} \) represents the ensemble level reaction rate constant. \( N_{eff} \) is the ratio of active Au nanoparticles and is included in the rate expression as we observe different TOFs between single molecule and ensemble level measurements. By dividing Eq. 14 on both sides by \( C_{NP} \), we obtain the conventional TOF.
From Figures 2.5 and 2.6 A, we observe that at 25 °C, the value of single molecule <TOF> approaches saturation value at 0.283 s\(^{-1}\) and ensemble level TOF\(_{\text{obs}}\) (at \(C_{\text{NP}} = 83 \text{nM}\)) attains saturation value at 0.074 s\(^{-1}\). A comparison of the data at the single molecule level and ensemble level suggests that the observed TOF is an order of magnitude lower at the ensemble level. This can be addressed by determining the value of \(N_{\text{eff}}\) in an ensemble level measurement. The value of coefficient of Eq. 15 (\(C_{\text{NP}} k_{r,\text{ens}} n_{1T} n_{2T} N_{\text{eff}}\)) is 0.074 and represents the value of TOF\(_{\text{obs}}\) as the value of \(k_{r,\text{ens}} n_{1T} n_{2T} N_{\text{eff}}\) is dimensionless and approaches unity. The value of \(k_{r,\text{ens}} n_{1T} n_{2T}\) should ideally be equal to the value of \(k_{r,\text{SM}}\) when every Au nanoparticle is active. Therefore, the value of \(N_{\text{eff}}\) is 0.261 (0.074/0.283) and this indicates that 26.1% of the Au nanoparticles are active in bulk. The reduced percentage of active Au nanoparticles in the bulk is responsible for the order of magnitude difference between the two modes of measurements. The experimental data in Figures 3A-C were fit using Eq. 14 using a weighted least-square method, yielding \(k_{r,\text{ens}} = 0.31 \text{ s}^{-1}\), \(K_A = 6.9 \times 10^6 \text{ M}^{-1}\), \(K_B = 2.9 \times 10^6 \text{ M}^{-1}\), \(K_{W1} = K_{W2} = 0.47 \text{ M}^{-1}\). The obtained kinetic parameters at the ensemble-level are similar to the ones obtained at the single-molecule level.

Although the ensemble-level kinetics is complex due to the reaction order dependence on Au NPs, the consistent kinetic values obtained between the single-molecule and ensemble levels confirm that a unifying reaction mechanism is possible. The ensemble-level reaction mechanism for the reduction of resazurin to resorufin on Au NPs has not been reported previously. In a separate study on catalytic Pt NPs for the conversion of resazurin to resorufin in the presence of \(\text{N}_2\text{H}_4\), Han et al.\(^{26}\) reported a non-linear dependence of initial rate
on Pt NP concentration at the ensemble-level. This observation is contradictory to their initial assumption that the reaction rate should display first-order dependence on NP concentration. Based on their initial assumption, the reaction rate, when normalized by NP concentration, should display zero-order dependence with NP concentration. This apparent difference between their assumption and observed result led to their conclusion that the determination of catalytic reaction kinetics is complex at the ensemble-level and masked by significant particle-particle interactions. In contrast, our ensemble-level measurements indicate second-order dependence of reaction rate on Au NP concentration. If our initial assumption of non-competitive adsorption between resazurin and NH$_2$OH is valid, we should expect that, when normalized by Au NP concentration, the reaction rate should display first-order dependence on Au NP concentration in the absence of particle-particle interactions. When normalized by Au NP concentration, the initial rates display first-order dependence on Au NP concentration (Figure 2.6C). The dependence of rates on NP concentration is defined by the underlying reaction kinetics and our results indicate that the reaction kinetics at the ensemble-level is not complicated by particle-particle interactions. We believe the real reason for the apparent differences in the rate between single-molecule and ensemble-level measurements is two-fold: the order of magnitude difference in reaction rates is related to the percentage of active Au nanoparticles while the lag in reactivity is most likely related to the reduction of Au active sites on an active Au nanoparticle due to the influence of the solvent. At the single-molecule level, the ability to observe catalytic turnover events indicates that the reaction happens at the active-site of an individual Au NP. This is most likely possible when the solvent molecule desorbs/removed from the Au NP surface, thereby allowing for the reactants to interact with the Au surface, i.e., the biased nature of single-molecule
measurements. In the case of ensemble-level measurements, our proposed mechanism highlights competitive adsorption between the reactants and the solvent molecules on the Au surface. To further illustrate that lag in reactivity observed at the ensemble-level is due to solvent inhibitory effects, the value of $K_w C_w$ is $\approx 25$ and is of the same order of magnitude as the observed difference in rates (factor of $\approx 10$) between the two modes of measurements. However, the proposed mechanism at the single-molecule and ensemble level must be verified for the influence of solvent (i.e., $K_w C_w$ inclusion versus exclusion of $K_w C_w$) and for kinetic and thermodynamic consistency.
Figure 2.5. (A, B) Resazurin concentration dependence of (A) $\langle \tau_{\text{off}} \rangle^{-1}$ and (B) $\langle \tau_{\text{on}} \rangle^{-1}$ at 20, 25, 35, and 45 °C. Each data point, at any given resazurin concentration, is collected from the same 5.4 nm Au nanoparticle irrespective of the indicated temperature. All experiments are in 1 mM NH$_2$OH. Solid lines are fits of the corresponding rate expressions for $\langle \tau_{\text{off}} \rangle^{-1}$ and $\langle \tau_{\text{on}} \rangle^{-1}$ (Eqns. (9) and (11), respectively) and the corresponding kinetic parameters $k_{r,SM}$, $K_A$ (A) and $k_d$ (B) are provided in the Table 2.1. All error bars in the graphs are a result of the average values from 10 Au NPs, except at 4 μM resazurin for which the error bars are determined from average values from 50 Au NPs.
Figure 2.6. Ensemble-level kinetics of reduction of resazurin to resorufin catalyzed by Au NPs in the presence of NH\textsubscript{2}OH. (A) Dependence of resazurin concentration on the initial reaction rate at 20, 25, 35, and 45 °C. Experiments were carried out at [NH\textsubscript{2}OH] = 1 mM. (B) Dependence of NH\textsubscript{2}OH concentration on the initial reaction rate at [resazurin] = 4 μM and at a temperature of 25 °C. (C) Dependence of Au NP concentration on the initial reaction rate at [resazurin] = 4 μM, [NH\textsubscript{2}OH] = 1 mM and at a temperature of 25 °C. The data in (A-C) was normalized to the Au NP concentration. Solid lines in (A-C) are fits to the rate expression for resazurin (-r\textsubscript{A}) provided by Eq. (14). The corresponding rate parameters are presented in Table 2.1. Inset: Dependence of ln (initial rate) on ln (Au NP concentration). Solid line is a linear fit and the slope of 2.08 corresponds to the second-order dependence of the reaction rate on [Au NP].
Table 2.1. Optimized rate parameters in Eq. (9) and (11) for resazurin reduction reaction on individual 5.4 nm Au nanoparticles

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Single-molecule Level</th>
<th>Ensemble Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{r,SM}$ (s$^{-1}$)</td>
<td>$K_A$ (μM$^{-1}$)</td>
</tr>
<tr>
<td>20</td>
<td>0.26 ± 0.01</td>
<td>7.88 ± 1.58</td>
</tr>
<tr>
<td>25</td>
<td>0.31 ± 0.01</td>
<td>6.90 ± 1.23</td>
</tr>
<tr>
<td>35</td>
<td>0.38 ± 0.01</td>
<td>5.96 ± 0.89</td>
</tr>
<tr>
<td>45</td>
<td>0.49 ± 0.01</td>
<td>5.15 ± 0.75</td>
</tr>
</tbody>
</table>

Ensemble-level at 25 °C

\[ K_B = 2.9 \, \mu M^{-1} \]

\[ n_1 n_2 = 1.7 \times 10^3 \]

\[ K_{W1} = 0.47 \, M^{-1} \]

\[ K_{W2} = 0.47 \, M^{-1} \]

\[ N_{eff} = 0.261 \]
Figure 2.7. Resazurin concentration dependence of (A) $\langle \tau_{\text{off}} \rangle^{-1}$ and (B) $\langle \tau_{\text{on}} \rangle^{-1}$ at 25 °C from three single 5.4 nm Au NPs demonstrating three different saturation levels. Solid lines in (A) and (B) are simulations of Eq. (3) and Eq. (6), respectively: at 25 °C, for particle 1, $k_{r,SM} = 0.27 \pm 0.01$ s$^{-1}$, $K_A = (6.9 \pm 1.1) \times 10^6$ M$^{-1}$, and $k_d = 1.27 \pm 0.1$ s$^{-1}$; for particle 2, $k_{r,SM} = 0.46 \pm 0.01$ s$^{-1}$, $K_A = (7.6 \pm 1.4) \times 10^6$ M$^{-1}$, and $k_d = 1.32 \pm 0.1$ s$^{-1}$; for particle 3, $k_{r,SM} = 0.35 \pm 0.003$ s$^{-1}$, $K_A = (7.2 \pm 1.2) \times 10^6$ M$^{-1}$, and $k_d = 1.33 \pm 0.09$ s$^{-1}$. All experiments are carried out in 1 mM NH$_2$OH.
2.3.3. Influence of solvent on the observed catalytic activity of Au NPs at the single-molecule and the ensemble level

Comparative studies between single-molecule and ensemble-level measurements suggest that the presence of water causes an inhibitory effect on the observed reaction rate at the ensemble-level but not at the single-molecule level because of the biased nature of the experimental observations. The influence of solvent can be better understood by carrying out the resazurin reduction reaction in the presence of heavy water (D$_2$O). The energy required to break a hydrogen bond in D$_2$O (1.56 kcal/mol) is about 0.24 kcal/mol higher than for H$_2$O (1.32 kcal/mol). This relates to stronger hydrogen bonding and greater structural order in liquid D$_2$O at a given temperature than in liquid H$_2$O.\textsuperscript{32} If the presence of D$_2$O results in decreased competition for sites between resazurin and the solvent, one would expect a decrease in the reaction rate which would correlate with a decrease in the value of $K_A$. At the ensemble-level, we determined the dependence on reaction rate on resazurin concentration in the presence of excess ND$_2$OD d$_3$-DCl and 5.4 nm Au NPs suspended in D$_2$O at 25 °C (Figure 2.8A). By assuming that the equilibrium constant of ND$_2$OD ($K_{B,D2O}$) is similar to the value of $K_B$ and by fitting the data to Eq. (14), we obtained $k_{r,ens} = 0.22 \pm 0.02$ s$^{-1}$ and $K_A = 4.4 \pm 0.9$ μM$^{-1}$; a decrease of 30% when compared to the value of $k_{r,ens}$ and $K_A$ in water. However, the equilibrium constant for D$_2$O ($K_D$) = 0.42 M$^{-1}$ was found to be approximately equal to the value of $K_W$. Even though $K_D$ and $K_W$ are small, the concentration of H$_2$O and D$_2$O are huge (≈ 55 M) and this demonstrates that the solvent dominates the surface coverage ($\theta_{H2O} \approx \theta_{D2O} \approx 0.96$) at the ensemble-level. These kinetic isotope results at the ensemble-level indicates that the presence of D$_2$O inhibits the binding of resazurin to the Au NP surface to a
greater extent than \( \text{H}_2\text{O} \) and results in decreased \textit{competitive} adsorption between the reactant and the solvent for the same active site on the Au surface.

The influence of solvent was also verified at the single-molecule level by determining the dependence of \( \langle t_{\text{off}} \rangle^{-1} \) and \( \langle t_{\text{on}} \rangle^{-1} \) on resazurin concentration when \( \text{H}_2\text{O} \) is replaced with \( \text{D}_2\text{O} \) at 25 °C (Figure 2.8B-C). By fitting the data to Eqs. (9) and (11), we obtained \( k_{r,SM} = 0.3 \pm 0.01 \text{ s}^{-1} \), \( K_A = 6.6 \pm 1.1 \mu\text{M}^{-1} \), and \( k_d = 1.2 \pm 0.05 \text{ s}^{-1} \); a decrease of less than 5% when compared to the values of \( k_{r,SM} \), \( K_A \), and \( k_d \) obtained in water. The observed difference is considered to be small and acceptable within the error limits attributed to experimental errors considering the complexity in performing single-molecule measurements. This further supports our proposed reaction mechanism that differences in the reactivity between the ensemble-level and the single-molecule level is most likely due to inhibition of active sites on Au nanoparticles by water. By examining the influence of solvent at the single-molecule and ensemble level, this study exemplifies the power of single-molecule approach wherein one can understand a reaction system by negating the influence of solvent; a phenomenon that is masked during ensemble-level measurements.
Figure 2.8. (A) Dependence of resazurin concentration on the initial reaction rate at 25 °C in the presence of D$_2$O. Solid lines are fits to the rate expression for resazurin ($-r_d$) provided by Eq. (14) with $k_{r,ens} = 0.22 \pm 0.02$ s$^{-1}$, $K_f = 4.4 \pm 0.9$ μM$^{-1}$ and $K_D = 0.42$ M$^{-1}$ in D$_2$O. These values are 30% lower than the values obtained in H$_2$O (Table 2.1). (B, C) Resazurin concentration dependence of (B) $<\tau_{off}>^{-1}$ and (C) $<\tau_{on}>^{-1}$ at 25 °C in the presence of D$_2$O. Solid lines are fits to the rate expressions provided by Eq. (9) for (B) and Eq. (11) for (C) and resulted in $k_{r,SM} = 0.3 \pm 0.01$ s$^{-1}$ and $K_f = 6.6 \pm 1.1$ μM$^{-1}$ (B), and $k_d = 1.2 \pm 0.05$ s$^{-1}$ (C). These values are less than 5% lower than the values obtained in H$_2$O (Table 2.1). All experiments are in 1 mM NH$_2$OH.
2.3.4. Kinetic and thermodynamic consistency analysis of proposed reaction mechanism at the single-molecule and ensemble level

According to classical chemical kinetics, the standard procedure to verify a proposed reaction mechanism for kinetic and thermodynamic consistency is to carry out temperature-dependent measurements.\textsuperscript{30} At the single-molecule level, we carried out the reduction of resorufin on the surface of the same 5.4 nm Au NP at four different temperatures (20, 25, 35, and 45 °C). By varying the concentration of resazurin, we evaluated $<\tau_{\text{off}}>^{-1}$ and $<\tau_{\text{on}}>^{-1}$ at each temperature. At all temperatures tested, we observe that both $<\tau_{\text{off}}>^{-1}$ and $<\tau_{\text{on}}>^{-1}$ follow resazurin dependent saturation kinetics (Figures 2.5A-B). Eqns. (9) and (11) were fit to the experimental data by employing a weighted least-square method to find the minimum of the sum of the squared deviations between observed and model calculations. The optimized parameters for the constants ($k_{r,SM}$, $K_A$, and $k_d$) at four temperatures are presented in Table 2.1. From the Au NP averaged results, with increasing temperature, the values for $k_{r,SM}$ and $k_d$ increases while $K_A$ decreases (Figure 2.9A). This is in accordance with kinetic and thermodynamic expectations. A plot of $\ln K_d$ versus $1000/T$ will yield the value for $\Delta S_{\text{ads}}$, the standard entropy of adsorption, and $\Delta H_{\text{ads}}$, the standard enthalpy of adsorption (Figure 2.9B). It is important to note that the obtained thermodynamic parameters are apparent or observed, and not intrinsic values. In order to support the proposed reaction mechanism, it is necessary that upon adsorption, a molecule cannot lose more entropy than it possesses (i.e., its absolute entropy) and therefore, the intrinsic $\Delta S$ due to adsorption must be negative and must have a magnitude smaller than its absolute entropy in the gas-phase $S_{\text{g}}^\circ$. Another less rigorous consideration indicates that the minimum value of intrinsic $\Delta S$ is approximately -10 cal/mol·K, which corresponds to the loss of approximately one degree of translational
freedom.\textsuperscript{27} Our results indicate that the observed $\Delta S_{ads}$ yielded positive values ($\Delta S_{ads} = 19.7 \pm 0.8 \text{ cal/mol·K}$) for the binding of resazurin under the experimental conditions. This observed $\Delta S_{ads}$ can include contributions from solvation and desolvation of the reactant molecules. Adsorption of the reactant molecules in the liquid-phase requires desolvation of the adsorbates and the Au NP surface, followed by adsorption, and resolvation of the adsorbate-surface species. It is also known that water desorbs rapidly from a Au surface because the \hbox{H$_2$O-H$_2$O} interaction is stronger than the \hbox{H$_2$O-Au} interaction.\textsuperscript{33} As the reactants bind to the surface of the Au NP, the highly structured water next to the active site on the Au NP collapses to bulk water thereby accounting for the apparent large overall gain in observed $\Delta S_{ads}$ values. Concurrently, the value of $\Delta H_{ads}$ must be negative, due to the exothermic nature of adsorption, and the true rate constant ($k_{r,SM}$) must display Arrhenius behavior. The value of $\Delta H_{ads}$ is -3.1 ± 0.1 kcal/mol, and this indicates that resazurin is weakly adsorbed and suggests resazurin is mobile on the Au surface. At the ensemble-level, we evaluated the temperature-dependence of the initial reaction rate on resazurin concentration (Figure 2.6A). The data was fit to Eq. (14) and the values of $k_{r,ens}$ and $K_A$ were found to be consistent with the values obtained from single-molecule measurements (Table 2.1). The kinetic and thermodynamic analysis of the rate parameters carried out by our temperature-dependent single-molecule and ensemble-level measurements demonstrates the consistency of the proposed reaction mechanism.
Figure 2.9. (A) Temperature-dependent distribution of kinetic parameters $k_{r,SM}$, and $K_A$ obtained from fits with (A), and $k_d$ from fits with (B) at 20, 25, 35, and 45 °C. The kinetic parameters $k_{r,SM}$, $K_A$, and $k_d$ are obtained from a single NP and represent the kinetic rate and equilibrium constants averaged over all the sites present on the nanoparticle at any given temperature. All error bars in the graphs are a result of the average values from 10 Au NPs, except at 4 μM resazurin for which the error bars are determined from average values from 50 Au NPs. (B) Van’t Hoff plot demonstrating the temperature dependence of $K_A$. Solid lines are linear fits and $\Delta H$ and $\Delta S$ can be determined from the slope and the intercept, respectively. Error bars are SEM. The values of obtained thermodynamic parameters are $\Delta H_{ads} = -3.1 \pm 0.1$ kcal/mol and $\Delta S_{ads} = 19.7 \pm 0.8$ cal/mol·K.
2.3.5. Kinetic dispersion of individual Au nanoparticles at the single-molecule level and its comparison with ensemble-level measurements

An inherent advantage of performing kinetic measurements at the single-molecule level is that it allows for simultaneous determination of reaction and desorption kinetic parameters. It is extremely difficult to deconvolute desorption from reaction through ensemble-level measurements. We investigated the distributions of $<\tau_{off}>^{-1}$ and $<\tau_{on}>^{-1}$ from each single NP trajectory. At saturating resazurin and NH$_2$OH concentrations, all catalytically active sites are occupied by reactants and $\theta_A = \theta_B \approx 1$. At this condition, $<\tau_{off}>^{-1}$ reduces to $k_{r,SM}$ and $<\tau_{on}>^{-1}$ reduces to Eq. (13). Both $f_{off}(\tau)$ and $f_{on}(\tau)$ reduce to single-exponential decay functions, $f_{off}(\tau) = k_{r,SM}e^{-k_{r,SM}\tau}$ and $f_{on}(\tau) = k_{r,SM}(1+k_d)^2 e^{-k_d}\left(k_d^{(1+k_d)(k_d+2k_{r,SM}^{-1})^{-1}}\right)$. Figure 2.10A-B represent the distribution of $\tau_{off}$ and $\tau_{on}$ from the catalytic turnover trajectory of a single 5.4 nm Au NP at 20 °C. The distributions of $\tau_{off}$ and $\tau_{on}$ from the catalytic turnover trajectory of a single 5.4 nm Au NP at 25, 35, and 45 °C are presented in Figure 2.11A-F. The exponential distribution is clearly observed and the fit in Figure 2.10A corresponds to $k_{r,SM}$. The value of $k_d$ can be determined from the fit in Figure 2.10B. We tracked multiple catalytic turnover trajectories (~1000) at different temperatures for each individual Au NP and determined the temperature-dependent value of $k_{r,SM}$ and $k_d$. By repeating this procedure for 50 Au NPs, we obtained the temperature-dependent distribution of $k_{r,SM}$ and $k_d$ (Figure 2.10A,B, insets). The broad distribution of $k_{r,SM}$ and $k_d$ at each temperature indicates the dispersion in kinetic parameters among individual Au NPs, which is impossible to obtain through ensemble-level measurements. It is
important to remember that $k_{r,SM} = kn_1Tn_2T$ and represents the catalytic rate constant of the product formation from all the catalytically active sites on an individual Au NPs. Therefore, the kinetic dispersion observed in the value of $k_{r,SM}$ from individual Au NPs could involve contributions from dispersion in the catalytic rate constant of one active site ($k$) or the dispersion in the total number of catalytically active sites ($n_1Tn_2T$) on a single Au NP. Our observations are consistent with the ones reported by Xu et al.\textsuperscript{14} For the same reaction, they reported that the dispersion observed in the overall catalytic rate constant is primarily due to contributions from $n_1Tn_2T$ and not from $k$, based on the determination of a heterogeneity index parameter. The distribution of surface atoms on a 5.4 nm Au NP was presented in our previous study.\textsuperscript{34} It is well known that individual Au NPs differ in their distributions of surface atoms present at corners, edges, or terraces and each active site differs in its reactivity.\textsuperscript{22} The present study does not allow us to probe the reactivity of each individual active site on an individual Au NP, however, the differences in the calculated kinetic parameters as a function of temperature between individual Au NPs highlights the highly dispersive kinetics that is prevalent among different NPs.
Figure 2.10. (A, B) Distributions of (A) $\tau_{\text{off}}$ and (B) $\tau_{\text{on}}$ from a single 5.4 nm Au NP trajectory at 20 °C. Solid lines in (A,B) are single-exponential fits corresponding to the value of $k_{r,SM}$ and $k_d$ respectively. Insets: (A) Distributions of $k_{r,SM}$ at 20, 25, 35, and 45 °C from 50 Au NPs; Solid lines are Gaussian fits with center at 0.25, 0.29, 0.36, and 0.46 s$^{-1}$ and FWHM of 0.02, 0.03, 0.04, 0.07 s$^{-1}$. (B) Distributions of $k_d$ at 20, 25, 35, and 45 °C from 50 Au NPs; Solid lines are Gaussian fits with center at 1.26, 1.29, 1.32, and 1.38 s$^{-1}$ and FWHM of 0.14, 0.16, 0.21, 0.29 s$^{-1}$. All experiments are carried out at 4 μM resazurin (saturating concentration) and 1 mM NH$_2$OH. Distribution of $\tau_{\text{off}}$ and $\tau_{\text{on}}$ from a single 5.4 nm Au NP trajectory at 25, 35, and 45 °C can be found in Figure 2.11.
Figure 2.11. Distributions of $\tau_{\text{off}}$ from an individual 5.4 nm Au NP trajectory at (A) 25 °C, (B) 35 °C, (C) 45 °C. Solid lines in (A-C) are single-exponential fits corresponding to the value of rate constant ($k_{r,SM}$). Distributions of $\tau_{on}$ from the same 5.4 ± 0.7 nm Au NP trajectory at (D) 25 °C, (E) 35 °C, (F) 45 °C. Solid lines in (D-F) are single-exponential fits corresponding to the value of rate constant ($k_d$). All experiments are carried out in 4 μM resazurin and 1 mM NH$_2$OH. Arrhenius analysis of $k_{r,SM}$ and $k_d$ was used to determine values of $E_{a,SM}$ and $E_{des}$ to be 4.9 ± 0.4 kcal/mol and 0.7 ± 0.04 kcal/mol, respectively.
Another significant advantage in performing temperature-dependent measurements at the single-molecule level is that they enable simultaneous determination of $E_a$ and $E_{\text{des}}$ through Arrhenius plots. Arrhenius analysis of $k_{r,sM}$ and $k_d$ for each individual Au NP resulted in an activation energy ($E_{a,SM}$) of $4.52 \pm 0.4$ kcal/mol and a desorption energy ($E_{\text{des}}$) of $0.5 \pm 0.02$ kcal/mol (Figure 2.12A). The values of $E_{a,SM}$ and $E_{\text{des}}$ are statistically accurate due to the large number of turnover events (~1000) observed in order to obtain the distributions at any given temperature. The distribution of $E_{a,SM}$ and $E_{\text{des}}$ show significant heterogeneity among individual Au NPs (Figure 2.12B-C), both can be fitted using a Gaussian function, which yields average values of $E_{a,SM} = 4.6 \pm 0.4$ kcal/mol and $E_{\text{des}} = 0.4 \pm 0.02$ kcal/mol. Although the information related to desorption is lost during ensemble-level measurements, we anticipate the average values of $E_{a,SM}$ measured from several individual 5.4 nm Au NPs will resemble the ensemble average. Towards this, we carried out temperature-dependent ensemble-level conversion of resazurin to resorufin at saturating conditions (Figure 2.13A-B). By the method of initial rates, we identified that the reaction follows pseudo-first order kinetics and obtain an apparent rate constant ($k_{\text{app}}$) (Figure 2.13C). Arrhenius analysis of $k_{\text{app}}$ results in the value of apparent activation energy, $E_{a,\text{app}} = 15.1 \pm 0.8$ kcal/mol (Figure 2.13D). This value of $E_{a,\text{app}}$ is approximately three times larger than the $E_{a,SM}$ value of 4.6 kcal/mol obtained from single-molecule measurements. We believe the difference in the values of $E_{a,\text{ens}}$ and $E_{a,\text{app}}$ can be addressed by looking more carefully into Eq. 14. The value of $k_{\text{app}}$ is given by,

$$k_{\text{app}} = k_{r,\text{ens}} n_{1T} n_{2T} \frac{K_A C_A}{1 + K_{W2} C_W + K_A C_A} \frac{K_B C_B}{1 + K_{W2} C_W + K_B C_B} C_{\text{NP}}^2$$

(16)

$$\frac{K_B C_B}{1 + K_{W2} C_W + K_B C_B} \approx 1$$

(17)
From Eqns. (16) and (17), we can identify that as a function of temperature, $k_{app}$ depends on both $k_{r,ens}$ and $K_A$. Therefore, the value of $E_{a,app}$ (15.1 kcal/mol) obtained by the pseudo first-order kinetic analysis is approximately equal to the sum of $E_{a,ens}$ and $-\Delta G$ (9.3 kcal/mol). By carrying out an Arrhenius analysis on the kinetic rate parameters obtained from the fits of Eq. 14 to the kinetic data observed in Figure 2.6A, we determine an ensemble-level activation energy ($E_{a,ens}$) of 4.6 ± 0.5 kcal/mol. This value is identical to $E_{a,SM}$ obtained using single-molecule analysis and further verifies the consistency of the proposed reaction mechanism.
**Figure 2.12.** (A) Temperature dependence of ln $k_{r,SM}$ and ln $k_d$ from Figure 2C. Solid lines are linear fits and the slopes correspond to single-molecule activation energy ($E_{a,SM}$) of 4.6 ± 0.4 kcal/mol and desorption energy ($E_{des}$) of 0.4 ± 0.02 kcal/mol. Each data point is an average from 50 Au NPs and the error bars are SD. Error bars are present in (A) but difficult to see because they are small. (B,C) Distributions of (A) $E_{a,SM}$ and (B) $E_{des}$. Each data point was evaluated from a temperature dependent study (20, 25, 35, and 45 °C) on an individual 5.4 nm Au NP. The entire plot in (B,C) contains data from 50 individual 5.4 nm Au NPs, each undergoing hundreds of catalytic turnovers at a given temperature. The distributions for both energies are broad, indicating heterogeneity among individual Au nanoparticles. Solid lines are Gaussian fits with center at 4.6 kcal/mol and FWHM of 0.27 kcal/mol (B), and center at 0.4 kcal/mol and FWHM of 0.19 kcal/mol (C). All experiments were carried out at saturating conditions of 4 μM resazurin and 1 mM NH$_2$OH.
Figure 2.13. (A) Absorbance profiles of resazurin at 600 nm as a function of temperature. (B) Absorbance profiles of resorufin at 570 nm. The absorbance profile of the reaction solution shows over time a decrease of the resazurin absorption at 600 nm (A) and an increase of the resorufin absorption at 570 nm (B). The shift in the wavelength of the Au plasmon band is small and not observable at low concentration of the Au NP solution. The absorbance data from the first 5 seconds was utilized to determine the initial rates. (C) The apparent first-order conversion of resazurin to resorufin on 5 nm Au nanoparticles at different temperatures. The slope corresponds to the value of the overall catalytic rate constant ($k_{app}$) for the reaction at a given temperature. All experiments are in 4 μM resazurin, 1 mM NH$_2$OH, and 83 nM Au NPs. (D) Temperature dependence of ln $k_{app}$. Solid line is a linear fit and the slope corresponds to an ensemble level apparent activation energy ($E_{a,app}$) = 15.1 ± 0.7 kcal/mol.
2.4. Conclusions

The reduction of resazurin to resorufin over 5.4 nm Au NPs was studied at the single-molecule and the ensemble level. At the single-molecule level, it was observed that both resorufin formation and its subsequent desorption followed saturation kinetics. At the ensemble-level, the dependence of reaction rate on both resazurin and NH$_2$OH concentration followed saturation kinetics, while the reaction rate followed a non-linear dependence with Au NP concentration. A unifying reaction mechanism based on the Langmuir-Hinshelwood formalism, which assumed non-competitive adsorption between resazurin and NH$_2$OH and competitive adsorption between water and the reactants onto the same type of Au sites, with the formation of resorufin assumed to be the rate-determining step, fit the kinetic data well. The magnitude of the observed difference in reaction rates between the single-molecule and ensemble level was attributed to reduced percentage of active Au nanoparticles in bulk while the lag in reactivity was attributed to the inhibition of Au surface sites by water molecules at the ensemble-level, while the presence of solvent did not affect the observed rates at the single-molecule level. The effect of solvent on the reaction rate was further verified by kinetic isotope experiments by replacing H$_2$O with D$_2$O. At the ensemble-level, the presence of D$_2$O was found to further inhibit the reaction rate by preventing the resazurin and ND$_2$OD molecules from interacting with the Au NP surface, while no significant change in rate was observed at the single-molecule level. The proposed reaction mechanism was verified for kinetic and thermodynamic consistency by temperature-dependent single-molecule and ensemble level measurements. Analysis of the parameters in the rate expression using thermodynamic guidelines highlighted that they contained physically meaningful values for enthalpy and entropy of adsorption. The distributions of the kinetic parameters demonstrated the highly dispersive nature of individual Au NPs. The temperature-dependent and solvent-
dependent single-molecule study presented here not only reveals dispersive nature of individual Au NPs and distinct solvent effects, but can also have broader impacts on future studies.

2.5. References


2.6. Appendix

2.6.1. Reduction of Resazurin to Resorufin – Derivation of Kinetic Mechanism

The reaction sequence for the reduction of resazurin to resorufin is provided as a set of elementary reactions. We will use the same set of elementary reactions to derive the rate of the reaction at the ensemble-level, and the probability density function $f(\tau)$ of the waiting times ($\tau_{\text{off}}$ and $\tau_{\text{on}}$), and of the rates of product formation ($<\tau_{\text{off}}>^{-1}$) and rate of product desorption ($<\tau_{\text{on}}>^{-1}$) at the single-molecule level. The primary assumption is that the two reactants (resazurin and NH$_2$OH) adsorb to different types of active sites (i.e., non-competitive adsorption) on the Au NP surface. The other assumptions include:

1. Resazurin and NH$_2$OH bind to the surface sites on the Au NP reversibly and fast adsorption equilibrium is maintained at all times.
2. The surface sites on the Au NP are independent and the reactant molecules are assumed to bind to different surface sites. Our single-molecule experiments limits us from distinguishing individual surface site on the Au NP, and the obtained kinetic parameters are averaged among different sites present on the same NP.
3. The product molecule, resorufin, dissociates at a faster rate when compared to the rate of product formation. This assumption is valid as we observe significantly longer waiting times for product formation ($\tau_{\text{off}}$) compared to the waiting times observed for product dissociation from the Au NP surface ($\tau_{\text{on}}$) in our single-molecule measurements.

The observed rate from single-molecule and ensemble-level measurements indicates the rate observed at the single-molecule level is an order of magnitude larger than the rate observed at the ensemble-level (upon normalization with Au NP concentration). We attribute
this change in the rate between both modes of measurements to competitive adsorption between solvent and reactants at the ensemble level. The reaction mechanism can be described by the same set of elementary reactions at both the single-molecule and ensemble levels.

2.6.1.1. Elementary Reactions

\[ A + S_1 \rightleftharpoons AS_1 \]
\[ W + S_1 \rightleftharpoons WS_1 \]
\[ B + S_2 \rightleftharpoons BS_2 \]
\[ W + S_2 \rightleftharpoons WS_2 \]

\[ AS_1 + BS_2 \xrightarrow{k_r} R_{on}S_1 + X S_2 \]

\[ R_{on}S_1 \xrightarrow{k_d} R_{off} + S_1 \]

Where,

\( A = \text{Resazurin} \)

\( S_1 = \text{Au site to which resazurin binds} \)

\( W = \text{Water} \)

\( B = \text{NH}_2\text{OH} \)

\( S_2 = \text{Au site to which NH}_2\text{OH binds} \)

\( R = \text{Resorufin} \)
$X$ = Oxidation products of NH$_2$OH (primarily N$_2$O and H$_2$O)

$K_A$, $K_{W1}$, $K_B$, and $K_{W2}$ are equilibrium constants, while $k_r$ and $k_d$ are the rate constants associated with formation and desorption of resorufin, respectively, from the Au NP surface. $R_{on}$ refers to the product molecule resorufin in the “on” state still bound to the surface of the Au NP. $R_{off}$ refers to the product molecule in the “off” state once it desorbs from the surface of the Au NP.

2.6.1.2. Ensemble Level

In UV-Vis ensemble-level measurements, we assume formation of resorufin ($P$) to be an irreversible rate determining step, the apparent overall rate of formation of resorufin is given by:

\[
\text{Apparent Overall rate} = k_{r,ens} AS_1 BS_2
\]

\[
AS_1 = n_{1T} C_{NP} \theta_A = \frac{n_{1T} C_{NP} K_A C_A}{1 + K_{W1} C_W + K_A C_A}
\]

\[
C_A = C_{A0} - AS_1
\]

\[
BS_2 = n_{2T} C_{NP} \theta_B = \frac{n_{2T} C_{NP} K_B C_B}{1 + K_{W2} C_W + K_B C_B}
\]

\[
C_B = C_{B0} - BS_2
\]

where $k_{r,ens}$ is the overall rate constant for the reaction at the ensemble-level. $n_{1T}$ and $n_{2T}$ are the total number of adsorption sites for resazurin and NH$_2$OH, respectively. $\theta_A$ and $\theta_B$ represent the fractional coverage of resazurin and NH$_2$OH. $K_A$ and $K_B$ are the adsorption equilibrium constants for resazurin and NH$_2$OH. $K_{W1}$ and $K_{W2}$ are the adsorption equilibrium constants for water on two different surface sites $S_1$ and $S_2$. $C_W$ represents the concentration of water. $C_{A0}$ and $C_{B0}$ represent the initial concentration of resazurin and NH$_2$OH. In the case
of ensemble-level measurements, the concentration of resazurin and NH$_2$OH at any given point in time is given by $C_A$ and $C_B$, respectively.

The rate of disappearance of resazurin is a function of concentration of resazurin, NH$_2$OH, and Au nanoparticles. The apparent rate for A is given by,

$$-r_A(C_A, C_B, C_{NP}) = k_{r,ens} n_{1T} n_{2T} N_{eff} \frac{K_{A,app} C_A}{1 + K_{A,app} C_A} \frac{K_B C_B}{1 + K_{W2} C_W + K_B C_B} C_{NP}^2$$  \hspace{1cm} S6

where $N_{eff}$ is the ratio of active Au nanoparticles and is included in the rate expression as we observe different TOFs between single molecule and ensemble level measurements. $K_{A,app}$ and $k_{A,app}$ are the apparent equilibrium constant and rate constant for resazurin, respectively, and takes the form:

$$K_{A,app} = \frac{K_A}{1 + K_{W1} C_W}$$  \hspace{1cm} S7

$$k_{A,app} = k_{r,ens} \frac{n_{1T} n_{2T} C_{NP}^2 K_B C_B}{1 + K_{W2} C_W + K_B C_B}$$  \hspace{1cm} S8

Therefore, the apparent rate for A can be written as,

$$-r_A = k_{A,app} \frac{K_{A,app} C_A}{1 + K_{A,app} C_A}$$  \hspace{1cm} S9

By dividing Eq. S7 by the concentration of Au nanoparticles ($C_{NP}$) on both sides, we obtain the observed TOF ($TOF_{obs}$).

$$TOF_{obs} = k_{r,ens} n_{1T} n_{2T} N_{eff} \frac{K_A C_A}{1 + K_{W1} C_W + K_A C_A} \frac{K_B C_B}{1 + K_{W2} C_W + K_B C_B} C_{NP}$$  \hspace{1cm} (S10)
The assumption of non-competitive adsorption is valid as we observe second order dependence of the reaction rate on Au NP concentration (Figure 3C in main text). If the adsorption between resazurin and NH$_2$OH was competitive, then we should have observed a first order dependence of the rate on Au NP concentration.

2.6.1.3. Single-Molecule Level

We have applied the Langmuir-Hinshelwood mechanism to analyze the reaction kinetics of resazurin reduction to resorufin at the single-molecule level and the assumptions have already been listed. Based on the elementary steps described, we can independently represent the rate of product formation ($<\tau_{\text{off}}>-1$) and the rate of product desorption ($<\tau_{\text{on}}>-1$), respectively.

Mechanism for rate of product formation $<\tau_{\text{off}}>-1$

$$AS_1 + BS_2 \xrightleftharpoons[k_{r,SM}]{k_d} R_{on}S_1 + XS_2$$

Mechanism for rate of product formation $<\tau_{\text{on}}>-1$

$$R_{on}S_1 \xrightarrow{k_d} R_{off} + S_1$$

$$AS'_1 + BS'_2 \xrightleftharpoons[k_{r,SM}]{k_d} R_{on}'S'_1 + XS'_2$$

2.6.1.3.1. Determination of $<\tau_{\text{off}}>-1$

In the case of single-molecule measurements, we still assume that the reactants (resazurin and NH$_2$OH) adsorb to different types of Au surface sites (i.e., non-competitive adsorption). We believe that for the reaction to proceed, it is necessary for the water molecules to desorb from the sites $S_i$ and $S_2$ to facilitate the adsorption of resazurin and
NH$_2$OH. This leads to the omission of the effect of water in the determination of the rates at the single-molecule level. In addition, at any given point in time, the concentration of resazurin and NH$_2$OH will correspond to their initial concentration ($C_{A0}$, $C_{B0}$). Based on the mechanism for $\tau_{off}^{-1}$ and from Langmuir-Hinshelwood reaction kinetics,

$$k_{com} = k_{r,SM} \theta_A \theta_B = k_{r,SM} \frac{K_A C_{A0}}{1 + K_A C_{A0}} \frac{K_B C_{B0}}{1 + K_B C_{B0}}$$  \hspace{1cm} \text{(S11)}$$

Here $k_{r,SM}$ represents the single-molecule rate constant of the reaction and is equal to the value of the ensemble-level rate constant, $k_{r,ens}$. $\theta_A$ and $\theta_B$ represent the fractional coverage of resazurin and NH$_2$OH. $K_A$ and $K_B$ are the adsorption equilibrium constants for resazurin and NH$_2$OH.

In conventional ensemble measurements, where the reaction rate is measured in a large population of Au nanoparticles in solution simultaneous, the kinetic rate equations for $\tau_{off}$ reaction are:

$$\frac{d[AS_1BS_2]}{dt} = -k_{com}[AS_1BS_2]$$  \hspace{1cm} \text{(S12)}$$

$$\frac{d[R_{on}]}{dt} = k_{com}[AS_1BS_2]$$  \hspace{1cm} \text{(S13)}$$

where $[AS_1BS_2]$ represent the concentration of Au NPs that do not carry a product molecule, and $[R_{on}]$ represent the concentration of Au NPs with a product molecule. In the case of single-molecule measurements, the concentrations of reactants A and B is a valid description, however, the concentration of one Au NP does not represent anything meaningful. Instead, it should be replaced by the probability of single nanoparticles. Therefore, the kinetic equations for $\tau_{off}$ (S11 and S12) can be modified as,
\[ \frac{dP_{AS_1BS_2}(t)}{dt} = -k_{com}P_{AS_1BS_2}(t) \quad \text{S14} \]

\[ \frac{dP_{Ron}(t)}{dt} = k_{com}P_{AS_1BS_2}(t) \quad \text{S15} \]

where \( P(t) \)'s represent the probabilities of locating a single gold nanoparticle in the states \( AS_1BS_2 \) and \( Ron \) at any given time \( t \). At the onset of the reaction (\( t = 0 \)), no product molecule is present and the initial conditions for solving the Equation S13 and S14 are,

\[ P_{AS_1BS_2}(0) = 1, \quad P_{Ron}(0) = 0 \]

At any given time \( \tau_{off} \) during the course of the reaction,

\[ P_{AS_1BS_2}(\tau) + P_{Ron}(\tau) = 1 \]

We can now evaluate the probability density \( f_{off}(\tau) \) of the time \( \tau \) required to complete the reaction (formation of a product molecule). The probability of finding a particular \( \tau \) is \( f_{off}(\tau)\,d\tau \), which is equal to the probability of switching from the \( AS_1BS_2 \) state (off-state) to the \( PS_1S_2 \) state (on-state) between \( t = \tau \) and \( t = \tau + d\tau \).

\[ f_{off}(\tau) = \frac{dP_{Ron}(\tau)}{d\tau} = k_{com}P_{AS_1BS_2}(\tau) \quad \text{S16} \]

Solving Equations S14 and S15 for \( P_{AS1BS2}(\tau) \) by Laplace Transform and the specified initial conditions, we obtain:

\[ f_{off}(\tau) = k_{com}e^{-k_{com}\tau} \quad \text{S17} \]

Then, \( <\tau_{off}>^{-1} \), which represents the time averaged rate of product formation for a single nanoparticle is given by,

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\[
\langle \tau_{off} \rangle^{-1} = \frac{1}{\int_0^\infty \tau f_{off}(\tau) d\tau} = k_{com}
\]

We know that from Equation S11,

\[
k_{com} = k_{r,SM} \frac{K_A C_A}{1 + K_A C_A} \frac{K_B C_B}{1 + K_B C_B}
\]

Therefore,

\[
\langle \tau_{off} \rangle^{-1} = \frac{1}{\int_0^\infty \tau f_{off}(\tau) d\tau} = k_{r,SM} \frac{K_A C_A}{1 + K_A C_A} \frac{K_B C_B}{1 + K_B C_B}
\]

This equation is represented as Equation (9) in the main text. From this equation, \( \langle \tau_{off} \rangle^{-1} \) should increase with increasing concentration of reactants and eventually saturate toward \( k_{r,SM} \).

2.6.1.3.2. Determination of \( \langle \tau_{on} \rangle^{-1} \)

Based on the mechanism for \( \langle \tau_{on} \rangle^{-1} \), the on-time encompasses the time from product formation to product dissociation from the NP surface. Under the assumptions of Langmuir-Hinshelwood mechanism, we write the conventional kinetic rate equations in concentration terms:

\[
\frac{d[R_{off}]}{dt} = k_d [R_{on}]
\]

\[
\frac{d[R_{on}]}{dt} = -k_d [R_{on}] + k_{com} [S_1'S_2'AB]_{R_{off}}
\]

\[
\frac{d[S_1'S_2'AB]}{dt} = -k_{com} [S_1'S_2'AB]
\]
In Equation S19, the first term in RHS represent the rate of desorption of product and the second term in RHS represents rate of generation of product at different reacting sites after the desorption of product. If we replace the concentration terms of nanoparticles with their probabilities in Equations (S19 – S21), we obtain:

$$\frac{dP_{R_{off}}(t)}{dt} = k_d P_{R_{on}}(t)$$ \hspace{1cm} S22

$$\frac{dP_{R_{on}}(t)}{dt} = -k_d P_{R_{on}}(t) + k_{com} P_{S_1' S_2' AB} (1 - P_{R_{on}}(t))$$ \hspace{1cm} S23

$$\frac{dP_{S_1' S_2' AB}(t)}{dt} = -k_{com} P_{S_1' S_2' AB}(t)$$ \hspace{1cm} S24

The initial conditions for solving Equations (S22 – S24) are given by,

$$P_{R_{on}}(0) = 1, P_{R_{off}}(0) = 0, P_{S_1' S_2' AB}(0) = 1$$

We can now evaluate the probability density $f_{on}(\tau)$ of the time $\tau$ required for the product molecule to desorb from the Au nanoparticle surface. The probability of finding a particular $\tau$ is $f_{on}(\tau) \, \text{d}\tau$, which is equal to the probability of switching from the $R_{on}$ state (on-state) to the $R_{off}$ state or $S_1' S_2' AB$ (off-state) between $t = \tau$ and $t = \tau + \text{d}\tau$.

$$f_{on}(\tau) = \frac{dP_{R_{off}}(\tau)}{d\tau} = k_{d} P_{R_{on}}(\tau)$$ \hspace{1cm} S25

Solving Equations (S22 – S24) for $P_{S_1 S_2}$ ($\tau$) by Laplace Transform with the initial conditions, we obtain:

$$f_{on}(\tau) = k_{d} \frac{e^{\alpha \tau}(k_{d} + 2\beta - 3k_{app})\sinh(-\beta \tau)}{2\beta}$$ \hspace{1cm} S26
Where,

\[ \alpha = \frac{k_d + k_{com}}{2} \]

\[ \beta = \frac{\sqrt{(k_d - k_{com})^2 - 4k_{com}}}{2} \]

Then, \( <\tau_{on}>^{-1} \), which represents the time averaged rate of product desorption for a single nanoparticle is given by,

\[ <\tau_{on}>^{-1} = \frac{1}{\int_0^\infty \tau_{on}(\tau) d\tau} = \frac{k_{com}(1 + k_d)^2}{k_d(k_d + 2k_{com} - 1)} \]  

S27

This Equation is represented as Equation (12) in the main text. To give a physical interpretation of \( <\tau_{on}>^{-1} \), at saturating reactant concentrations, Equation S26 reduces to,

\[ <\tau_{on}>^{-1} = \frac{k_{r,SM}(1 + k_d)^2}{k_d(k_d + 2k_{r,SM} - 1)} \]  

S28

The value of desorption rate constant \( (k_d) \) can be determined by substituting the value for reaction rate constant \( (k_{r,SM}) \) in Equation S28.

\[ k_d = \frac{-2k_{r,SM} - <\tau_{on}>^{-1} + 2k_{r,SM}<\tau_{on}>^{-1} - \sqrt{(2k_{r,SM} - <\tau_{on}>^{-1})(8k_{r,SM} - 2k_{r,SM}^2 + <\tau_{on}>^{-1} - 4k_{r,SM}<\tau_{on}>^{-1} + 4k_{r,SM}^2<\tau_{on}>^{-1})}}{2(k_{r,SM} - <\tau_{on}>^{-1})} \]

Performing experiments at the single-molecule level will allow us to determine the kinetic rate parameters associated with desorption of the product molecule even when desorption is not the rate limiting step.
Chapter 3

Modulating Static and Dynamic Variations in Activity of Individual
Catalytic Nanoparticles Probed at the Single Molecule Level

3.1. Introduction

Nanoparticles (NPs) are intrinsically heterogeneous in nature and are predominant catalytic entities in the advancement of future energy technologies.\(^1\)\(^-\)\(^4\) The catalytic surface of a nanoparticle during reaction is not a static entity, but rather a dynamic entity in which strong adsorption or heat release associated with an exothermic surface reaction can give rise to dynamic change of surface structure.\(^3\) The act of adsorption by strong adsorbates results in either reversible or permanent changes (restructuring) in the catalyst surface structure and the unstable nature of the surface under reaction conditions also contributes to the dynamic behavior.\(^5\) Gold nanoparticles (Au NPs) are active heterogeneous catalysts in both gas and liquid phases, and find wide applications in catalyzing reactions that occurs in pollution control, chemical processing, and fuel cell technology.\(^6\)\(^-\)\(^11\) Recently, Chen and co-workers observed variations in activity between individual catalytic turnover events over individual Au NPs (6-13 nm) during the catalytic reduction of resazurin using a single-molecule fluorescence approach.\(^12\)\(^-\)\(^17\) They proposed this variation in activity is a result of NP restructuring between individual turnover events. It is important to understand that regardless of the origins of this restructuring behavior, it must manifest itself over the same timescale as a catalytic turnover event in order to influence individual turnovers. This time-dependent and particle-dependent variation in activity can only be observed at the single NP level with a method that has single turnover resolution. It is difficult to probe the variation in activity at
the ensemble-level since catalytic turnover is asynchronous in time over a population (ensemble) of NPs.

Single-molecule measurements have become an extremely powerful tool for studying biological and biophysical phenomena over the past two decades. Single-molecule studies have their foundation in enzymology since enzymes were found to be dynamic entities which exhibit constant variations of catalytic rate constants. They have shed light on the variation in reactivity among individual enzymes (static variation) and shown that temporal variations in the reaction rate exist for single enzymes (dynamic variation). The origins of the dynamic variation has been ascribed to conformational changes of the enzymes in the absence of any adsorbate/reaction (intrinsic restructuring) and the surface reconstruction due to adsorbate binding/unbinding events and the catalytic reaction (adsorbate-induced restructuring). Over the past decade, we have seen the single-molecule approach applied to the understanding of catalytic behavior of heterogeneous catalysts. Recent studies of ester hydrolysis on the surface of a layered double hydroxide crystal and the epoxidation of phenylbutadienyl-substituted boron dipyrromethene difluoride (PBD-bodipy) on micron-sized Ti-MCM-41 particles have shown the ability to measure individual turnovers in real-time and probe the variations in activity that exist among individual turnover events. Chen and co-workers have reported that temporal variations in activity among large individual Au NPs are a result of intrinsic and adsorbate/catalysis-induced restructuring. Furthermore, they identify that both intrinsic restructuring and catalysis/adsorbate-induced restructuring occur more readily for smaller sized Au NPs (6.1 nm diameter) due to their higher surface energies. However, unlike enzymes, an individual NP has a number of active sites that behave differently but are indistinguishable from one
another with the current spatial resolution available. When considered on a nanoparticle basis, it is extremely difficult to deconvolute *dynamic variation* and *static variation*. One possible method to understand the contributions of *dynamic variation* and *static variation* to the observed variations in activity is to examine its dependence on intrinsic properties of a catalytic NP. The critical properties that are related to the catalytic activity of NP are its size, shape, type and number of active sites on a NP. In addition, the NPs are capable of structural transformations during reactions that can also contribute to its observed activity.

If the real reason for *dynamic variations* in activity is due to the restructuring ability of a NP, then it could be directly probed by suitable experimental or computational techniques. At present, there are no experimental methods to directly determine the contribution of restructuring to the observed *dynamic variations* in activity. We aim to address the differences between *static* and *dynamic* variations in activity by coupling Reaction Force-Field (ReaxFF) Molecular Dynamics (MD) simulations with the experimental examination of the catalytic activity of an individual Au NPs (5-20 nm) using the resazurin reduction to resorufin as a model fluorescent reaction capable of single-molecule detection. Systems capable of restructuring, due to their small cohesive energy, are influenced by any perturbation: ligands, solvents, and even by the presence of inert gas atmosphere. We aim to understand the dependence of intrinsic reactivity of different types of active sites on the surface of a Au NP on the observed variations among individual turnover events by utilizing small molecule adsorbates (carboxylic acid-terminated thiols and amines) of varying affinity to the Au surface. Furthermore, we attempt to effectively modulate the variations in activity on individual Au NPs by manipulating the Au surface coverage using these small molecule adsorbates.
3.2. Experimental

3.2.1. Materials

Thioglycolic acid (C\textsubscript{2}-TGA), 3-mercaptopropionic acid (C\textsubscript{3}-MPA), 6-mercaptophexanoic acid (C\textsubscript{6}-MHA), 11-mercaptopropionic acid (C\textsubscript{11}-MUA), and 3-aminopropionic acid (β-alanine) were obtained from Sigma Aldrich (Milwaukee, WI). Gold nanoparticles (5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm) protected by citrate in aqueous dispersions were obtained from Ted Pella (Redding, CA). Milli-Q grade water (18.2 M\textOmega\cdot \text{cm}) was used in all experiments. All chemicals were used as received.

3.2.2. Single nanoparticle experiments

Single molecule experiments were performed on an Olympus IX-71 total internal reflection fluorescence (TIRF) microscope. The microscope is illuminated using a CW Argon/Krypton-ion laser (35-KAP-431-220, Melles Griot, Carlsbad, California, USA) with 476 to 676 nm excitation; output power 4 to 20 mW. A CW vertically-polarized 520 nm beam was focused on the sample for the fluorescence excitation of resorufin. An oil immersion objective (PlanAPO 60×/1.45 NA, TIRFM-2, WD 0.15 mm) was used for collection of the fluorescent photons. For TIRF, laser light is coupled via a fiber-optic cable to the TIRF illuminator on one end of the epiport and focused off-center on the back aperture of the objective. A Peltier-cooled 12-bit digital CCD camera (SensicamQE) with high resolution, sensitivity, and high quantum efficiency (65%) in the visible range was used to detect photons emitted by resorufin. The camera was modified with a fast shutter to enable the collection of two successive images with an interval of 500 ns. The camera was operated at 45 msec time resolution for all the single molecule experiments. Movies of fluorescence
bursts were analyzed using a home-written MATLAB program, which extracts the individual fluorescence intensity trajectories from localized fluorescent spots over the entire duration of the movie.

3.2.3. Construction of the flow reactor

In order to measure the individual fluorescence bursts due to the production of a single molecule of resorufin, we interfaced a homemade flow reactor with the TIRF microscope. The flow cell is made from a glass slide and a glass coverslip; the bottom glass coverslip is functionalized with 3-aminopropyltriethoxysilane (APTES) in acetone and the citrate-stabilized Au nanoparticles are electrostatically adsorbed on this surface. The citrate-covered Au nanoparticles were then ligand-exchanged (0-100%) with thiols (C2-TGA, C3-MPA, C6-MHA, and C11-MUA) and amines (β-alanine). The cover slip was rinsed with Milli-Q water to remove unbound Au NPs and thiol molecules. Holes were drilled on the top glass slide and are fitted with polyethylene tubing for the continuous passage of aqueous resazurin/hydroxylamine solution from a syringe pump. The glass slide and the glass coverslip are bound to each other via a piece of 200 μm thick double-sided tape.

3.2.4. Isothermal Titration Calorimetry to determine strength of binding of reactants and small molecule adsorbates on Au nanoparticles

Isothermal titration calorimetry (ITC) experiments were performed using a NanoITC calorimeter (TA instruments) equipped with hastelloy reference and sample cells ($V = 1.014$ ml). All titrations were carried out at 25 °C using a 100 μL syringe at a stirring rate of 250 rpm. The sample cell contains an aqueous dispersion of 5.4 nm Au nanoparticles and the reference cell contains Milli-Q water. All solutions were degassed by pulling a vacuum of
0.3-0.5 atm using a temperature-controlled degassing system (TA instruments) for a period of 10-15 min prior to titrations. All titrations except the ones using thiols were run as one continuous injection of the appropriate solution into the gold nanoparticle solution, and the power compensation was measured as a function of time. The area under each peak is a measure of two effects: heat of mixing of the adsorbate and adsorbate binding to the surface of the nanoparticle. Experiments conducted under identical conditions with Milli-Q water (no Au NPs) in the sample cell yielded experimental values for the heat of mixing. The heat of mixing data was subtracted from the experiments with Au NPs in the cell to obtain the heat evolved from adsorbate-NP interactions. From the number of moles of adsorbate, we determined the observed heat of adsorption (ΔH). The value of $K$ was obtained by assuming a loss of one degree of translational freedom for the value of ΔS. In the case of thiols, titrations were run as an incremental series of injections of the appropriate thiol solution into the gold nanoparticle solution, and the power compensation was measured as a function of time. Data analysis was performed using the NanoAnalyze software from TA instruments using an independent model.31

3.2.5. Ensemble-level measurements of resazurin reduction by Au NPs in the presence of thiols and amines using UV-Vis Spectroscopy

We carried out ensemble-level kinetic measurements to determine the turnover frequency (TOF) for the reduction of resazurin to resorufin in excess NH$_2$OH catalyzed by 5.4 nm Au NPs at varying thiol and β-alanine coverage. The catalytic reactions were carried out using a Shimadzu UV-3600 UV-Vis-NIR spectrophotometer at 25 °C in water and were initiated by adding aliquots of a mixture of aqueous 5 nm Au nanoparticle and thiol solution into a premixed solution of resazurin and NH$_2$OH. The coverage of thiol (in % assuming a
1:3 S:Au₈ ratio, where S and Au₈ represents sulfur and Au surface atoms, respectively) and amines (in % assuming a 1:1 S:Au₈ ratio) was conducted batch-wise. With the known concentration of particles (5×10¹³ particles/ml) and the average size of the nanoparticles (5.4 nm), the concentration of Au₈ was determined and an appropriate amount of thiol/amine was added to a well-mixed solution to obtain a specified thiol/amine coverage. Upon injection of thiol/amine covered Au NPs, the solution gradually turns from blue (resazurin) to red (resorufin). Resazurin absorbs at 600 nm and resorufin absorbs at 570 nm. The instrument is set-up to record the absorbance at both 600 and 570 nm with a time resolution of 1 min. The absorbance data from the first 5 minutes was utilized in order to determine the initial reaction rate and the TOF in the absence of thiol. In the presence of thiols/amines, the absorbance data from the first 10 minutes was utilized in order to determine the initial reaction rate and the TOF as a function of thiol and amine coverage. The shift in the wavelength of the Au plasmon band is small and not observable at low concentration of the Au NP solution.

A control experiment demonstrated Au NPs were required for the reduction of resazurin by NH₂OH. Additionally, the reductant, NH₂OH was required for the reduction of resazurin over Au NPs. To convert the absorbance data into concentration, we determined extinction coefficients of both resazurin and resorufin. The extinction coefficient of resazurin at 600 nm was determined to be $\varepsilon_{600 \text{ nm}} = 32537 \text{ M}^{-1} \text{ cm}^{-1}$ and resorufin at 570 nm was $\varepsilon_{570 \text{ nm}} = 54242 \text{ M}^{-1} \text{ cm}^{-1}$.

3.3. Results and Discussion

3.3.1. Effect of intrinsic restructuring behavior of Au NPs on observed variations in activity
The catalytic activity of a nanoparticle is related to the dispersion or the ratio of the surface atoms to the total number of atoms in structure sensitive reactions.\textsuperscript{32,33} Surface atoms have a lower coordination number and are weakly bound to the nanoparticle. These surface atoms are susceptible to restructuring in the absence of any adsorption/reaction. A recent study by Vargas et al. confirms that intrinsic restructuring is displayed by Au clusters with 12-55 gold atoms in vacuum.\textsuperscript{34} All atoms on the cluster behave as surface atoms and exhibit intrinsic restructuring behavior. The formation of different equilibrium structures is dynamic with the longest lived structure being stable for \textasciitilde 9 ps.\textsuperscript{34} As the size of the NP increases, a distinct formation of core-shell structure occurs. The core atoms do not undergo any restructuring while the surface is susceptible to restructuring. This is understandable as catalysis is typically confined only to the surface of the Au NPs. We investigated the size-dependent restructuring of Au NPs (1, 2, and 5 nm diameter) utilizing reactive force-field (ReaxFF) molecular dynamics (MD) simulations.\textsuperscript{35} It is apparent from Figure 3.1 that restructuring is prevalent in small particles (1 nm) at 300 K over the 1 ns simulation. The 1 nm Au NP consists of 42 Au atoms and the intrinsic restructuring observed from our ReaxFF MD simulations are only from the Au surface atoms. The restructuring assessed through mean square displacement (MSD) of the Au surface atoms is highly sensitive to particle size; particles with a diameter of 2 nm displayed significantly reduced displacement; even at 500 K. The 5 nm Au NP did not exhibit any intrinsic restructuring behavior even at 800 K. Variations on a timescale commensurate with catalytic turnovers ($O$(sec)) cannot be observed by MD simulations, but the results in Figure 3.1 confirm particles with a diameter > 2 nm will experience minimal/no intrinsic restructuring.
ReaxFF calculations indicate the barrier between two isometric configurations of a 1
nm Au NP is 0.5 eV. This value is within the apparent barrier (1 eV) estimated from electron
microscopy for a particle to convert between different shapes.\textsuperscript{36} However, the barrier for a 2
nm Au NP was determined to be 1.4 eV. This is in agreement with the MSD calculations that
Au NPs > 2 nm do not exhibit any intrinsic restructuring at room temperature. This is in
contrast to the interpretations on intrinsic restructuring made by Zhou et al.\textsuperscript{16} By using a
thermodynamic model, they provided estimates for timescales of spontaneous/intrinsic
restructuring (60 – 250 s) for a particle range of 6 – 15 nm at room temperature. These Au
NPs (6-15 nm) are relatively large and their surface is comparable to surfaces of large single
crystals. Large platinum single crystals have been imaged under vacuum and it is reported
that the surfaces of these crystals are relatively static (thermodynamically stable) and do not
undergo any intrinsic restructuring.\textsuperscript{37} Although our ReaxFF MD simulations indicate the
absence of intrinsic restructuring on larger particles, these simulations have certain
limitations. The primary limitation is that the simulations are carried out in ns while
experimentally observed reaction timescales are on the order of seconds. The second
limitation is that the simulations are carried out in the absence of solvent. While there are
certain limitations to the ReaxFF MD simulations, recent developments have shown a new
class of simulations known as “accelerated dynamics methods” that are dedicated solving
these issues. They can attain simulation times of several orders of magnitude longer than
direct ReaxFF MD while retaining full atomistic detail. The methods include different classes
of simulations such as hyperdynamics, parallel replica dynamics, temperature-accelerated
dynamics, and on-the-fly Monte Carlo simulations.\textsuperscript{38} These methods are currently being
applied to understand metallic surface diffusion and surface growth and could potentially address the influence of intrinsic restructuring across similar timescales for reaction.\textsuperscript{39}
Figure 3.1. Mean square displacement (MSD) of Au atoms in a 1 and 2 nm Au NP determined from ReaxFF MD simulations for a maximum of 1 ns and MSD of Au atoms in 1 nm Au NP in the presence of varying coverage of methanethiol for a maximum of 1 ns. The Au nanoparticle (or Au-methanethiolate in the case of thiol MD simulations) geometry was energy-minimized to obtain a stable configuration at 0 K, followed by heating to the desired temperature in 50 K time-steps. All MD-NVT (Molecular Dynamics – constant number of atoms, constant volume, and constant temperature) simulations were carried out in vacuum at 300 or 500 K with a time step of 0.25 fs. Image on the right represents three representative isomers of the 42 atoms 1 nm Au NP observed during the simulation.
3.3.2. *Effect of adsorbate-induced restructuring and NP size on observed variations in activity*

To experimentally determine the influence of adsorbates and Au NP size on the variation in activity, we track individual catalytic turnover events on single 5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm Au NPs (Figure 3.2 represents Au NP size distribution) by monitoring the real-time reduction of resazurin to resorufin by $\text{NH}_2\text{OH}$. By maintaining a constant flow (5 μL/min) of resazurin (4 μM) and $\text{NH}_2\text{OH}$ (1mM), we measured individual fluorescent bursts due to the production of a single molecule of resorufin on the Au NP by interfacing a home-made cell with a TIRF microscope (Figure 3.3A). The individual turnovers are represented by photon bursts of arbitrary duration and “dark” periods, also of arbitrary length (Figure 3.3B). The “dark” period, also referred to as the waiting time between bursts ($\tau_{\text{off}}$), represents the time taken for the reactant molecules to diffuse through the boundary layer to the particle surface, adsorb on the Au surface, and react to form the product molecule. Once the product molecule is formed, the waiting time ($\tau_{\text{on}}$) represents the desorption behavior (direct measure of surface residence time) of resorufin. Addition of excess citrate to the solution (up to 100 mM) had no effect on the observed activity, i.e., the calculated rate, $<\text{rate}> = \left(<\tau_{\text{off}}> + <\tau_{\text{on}}>\right)^{-1}$ was found to be constant (Figure 3.4). Similar conclusions were reached by Xu et al.\textsuperscript{14}; the observed variations in activity is not caused by the citrate used to synthesize the Au NPs and the surface coverage of citrate is not a determining factor in observed variations in activity.
Figure 3.2. (A-C) TEM Micrographs of Au NPs used in our study and (D-F) represent their corresponding particle size distribution. From the particle size distribution, the average diameter of the Au NPs used in our study are (A, D) 5.4 ± 0.7 nm, (B, E) 9.5 ± 0.6 nm, and (C, F) 19.4 ± 1.1 nm respectively. The total number of Au nanoparticles used to determine the size distribution histograms are 347 for 5.4 nm, 202 for 9.5 nm, and 217 for 19.4 nm respectively. It is important to note that there is very little/almost no overlap between Au NPs.
Figure 3.3. (A) Experimental schematic of a flow reactor used to image catalytic turnover events on the surface of individual Au NPs. A glass cover slip is functionalized with 3-aminopropyltriethoxysilane (APTES) and citrate-stabilized 5.4 nm Au nanoparticles are electrostatically-adsorbed onto this surface. The reactant solution is passed on top of the Au NPs by means of polyethylene tubing connected to the top slide. The non-fluorescent resazurin is converted to the highly fluorescent resorufin when illuminated by a 520 nm laser. (B) A segment of a fluorescent trajectory of a single 5.4 nm Au NP for the conversion of resazurin to resorufin in the presence of NH$_2$OH at single turnover resolution. Embedded image of the bright spot represents the formation of a product molecule (on) and the blank image shows its desorption (off).
Figure 3.4. Values of $<\tau_{off}>^{-1}$ and $<\tau_{on}>^{-1}$ in the presence of excess of sodium citrate. The experiments were carried out 4 μM resazurin and 1 mM NH$_2$OH at 25 °C. Error bars are S.D.
Chen and co-workers have observed variations in activity over single Au NPs (6-14 nm) during single molecule studies of resazurin reduction. Through an autocorrelation analysis, they attributed the variations in activity to both intrinsic and adsorbate-induced particle restructuring.\textsuperscript{12,15,16} From our single-molecule trajectories, the observed variations in activity among each individual catalytic turnover events could be due to reaction rate changes in the product formation (\(\tau_{\text{off}}\)), the product dissociation (\(\tau_{\text{on}}\)), or both. To separate the contributions of \(\tau_{\text{off}}\) and \(\tau_{\text{on}}\) to the observed variations in activity, we extracted individual \(\tau_{\text{off}}\) and \(\tau_{\text{on}}\) values from a single trajectory, and calculated their autocorrelation function (\(r_k\)),

\[
\text{where } N \text{ is the number of observations in the whole series, } k \text{ is the lag, } Y \text{ is either } \tau_{\text{off}} \text{ or } \tau_{\text{on}}, \text{ and } \bar{Y} \text{ is the mean of the whole series and the denominator is the variance of the whole series. If events are random, the autocorrelations should be near zero for any and all time-lag separations. If events are non-random, one or more of the autocorrelations will be significantly non-zero (above the confidence interval limit).}\textsuperscript{40} \text{In the presence of correlations between individual turnover events, a value of } r_k \geq 0 \text{ shows a decay behavior with the decay time constant equal to the fluctuation correlation time.}\textsuperscript{22} \text{In the absence of correlations between individual turnover events, one would expect the value of } r_k \approx 0.\textsuperscript{22} \text{For one 5.4 nm Au NP, at saturating resazurin concentration (4 μM), } r_k \approx 0 \text{ and within the 95% confidence interval (C.I.), indicating individual values of } \tau_{\text{off}} \text{ and } \tau_{\text{on}} \text{ are uncorrelated and random in nature (Figure 3.5A-B). This procedure was repeated for 50 Au NPs and we observed no correlations between individual turnover events in either } \tau_{\text{off}} \text{ or } \tau_{\text{on}} \text{ values. If underlying restructuring is responsible for observed variations in activity, one would expect to observe}
correlations between individual turnover events as each turnover event can potentially take place on a different isomer of the Au NP. The absence of correlations between individual turnover events suggests that each turnover event most likely occurs on an identical Au NP equilibrium structure and the adsorption process does not lead to particle restructuring. Furthermore, from previous temperature-dependent measurements on the catalytic activity of Au NPs for the reduction of resazurin to resorufin and kinetic modeling, we determined the adsorption enthalpy of resazurin on 5.4 nm Au NPs is $-3.1 \pm 0.1$ kcal/mol.\textsuperscript{41} Further experimental verification of the adsorption enthalpy values was carried out using isothermal titration calorimetry (ITC). ITC experiments resulted in an adsorption enthalpy for resazurin and resorufin of 5.4 nm Au NP on the order of ~ (-1.0 to -1.5) ± 0.1 kcal/mol (Table 3.1). This suggests that resazurin and resorufin interacts very weakly with a solvated Au surface. Overall, the absence of correlations between individual turnover events, coupled with the weak interactions of the adsorbates towards the Au surface, indicates that adsorbate/catalysis-induced restructuring of Au NPs does not lead to the observed variations in activity for Au NPs $\geq 5$ nm in diameter at the single-particle level.
**Figure 3.5.** (A,B) ACF of (A) $\tau_{\text{off}}$ and (B) $\tau_{\text{on}}$ determined from a single 5.4 nm Au NP fluorescence trajectory. The dotted lines in (A,B) represent the 95% Confidence Intervals (C.I.) (C) Experimental determination of single molecule overall rate $\langle \text{rate} \rangle$ as a function of Au NP size. All experiments were carried out at 4 $\mu$M resazurin, 1 mM NH$_2$OH and 25 °C. Each data point was based on ~ 1000 individual catalytic turnover events. Error bars are S.D.
Table 3.1. Thermodynamic parameters derived from ITC data at 298 K.

<table>
<thead>
<tr>
<th>Adsorbate</th>
<th>$K$ (M$^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$T\Delta S^a$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resazurin</td>
<td>$4.8 \times 10^3$</td>
<td>-1.0</td>
<td>-3.0$^a$</td>
</tr>
<tr>
<td>Resorufin</td>
<td>$3.2 \times 10^4$</td>
<td>-1.4</td>
<td>-3.0$^a$</td>
</tr>
<tr>
<td>3-MPA$^b$</td>
<td>$3.3 \times 10^6$</td>
<td>-20.3$^b$</td>
<td>-11.4$^b$</td>
</tr>
<tr>
<td>$\beta$-alanine</td>
<td>$8.1 \times 10^3$</td>
<td>-0.8</td>
<td>-3.0$^a$</td>
</tr>
</tbody>
</table>

$^a$Assumed loss of one degree of translation freedom (~ 10 e.u.).

$^b$Measured from incremental titration of 3-MPA assuming non-interacting, one-site model.
We next determined the influence of Au NP size on the observed variations in activity. At a particle size of ~5.4 nm, the surface atoms approach bulk coordination and the speciation of various surface atoms types does not change significantly over this particle size range. We determined the overall rate of the reaction \(<rate>\) by observing multiple (~1000) catalytic turnover events on individual 5.4, 9.5, and 19.4 nm Au NPs (Figure 3.5C). Although variations in activity among individual turnovers exist, the observed \(<rate>\) was found to increase only by a factor of 1.5 as the particle size changes from 5.4 to 19.5 nm. If underlying restructuring of these Au NPs is the reason for the observed differences in activity, one would expect the \(<rate>\) to vary by a larger magnitude as a particle size changes. The \(<rate>\) for the reduction of resazurin appears to be insensitive to catalyst structure, i.e., the Au NP structure over the size range used in our experiments is invariant and an adsorption/reaction event does not lead to particle restructuring under reaction conditions. Instead, the observed variations in activity possibly indicates a greater probability for each individual turnover event to occur on a different active site with intrinsically different reactivity. The Au NPs used in our experiments are relatively large and expose primarily (111) facets, a small fraction of (100) facets and an even smaller fraction of under-coordinated sites such as edges and corners (Table 3.2). It has been determined that the cuboctahedral is the most stable shape for large Au nanoparticles (> 3.5 nm in diameter). According to the theory on the statistics of surface atoms of perfect cuboctahedron NPs by Van Hardeveld and Hartog, we determined the percentage of under-coordinated edges and corners for 5, 10, and 20 nm Au NPs as 10.1, 5.8, and 3.2%, respectively (Table 3.2). The under-coordinated sites are the most active on any nanoparticle as these sites present the lowest energy state. The 5 nm Au NP has the largest fraction of surface atoms that are
categorized as under-coordinated corners and edges, but the 20 nm Au NP has the largest absolute number of these types of sites (Table 3.2). As the particle size increases from 5.4 – 19.4 nm, the total number of surface atoms, absolute number of Au (100) atoms, and absolute number of Au (111) atoms increase by a factor of 13.9, 15.1, and 15, respectively. However, for the same particle size regime, the under-coordinated corners and edges increase only by a factor of 4.4 (Table 3.2). Therefore, the observed slight increase in <rate> as a function of NP size can be related to the change in the absolute number of under-coordinated sites.
### Table 3.2. Statistics of gold atom distribution for cuboctahedral Au particles

<table>
<thead>
<tr>
<th>Au NP dia (nm)</th>
<th>Total number of Au atoms</th>
<th>Au (111) surface atoms</th>
<th>Au (100) surface atoms</th>
<th>Au corner and edge atoms</th>
<th>% Au (111) surface atoms</th>
<th>% Au corner and edge atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4033</td>
<td>848</td>
<td>210</td>
<td>120</td>
<td>71.9</td>
<td>10.1</td>
</tr>
<tr>
<td>10</td>
<td>29881</td>
<td>3440</td>
<td>858</td>
<td>264</td>
<td>75.4</td>
<td>5.8</td>
</tr>
<tr>
<td>20</td>
<td>202746</td>
<td>12680</td>
<td>3168</td>
<td>528</td>
<td>77.4</td>
<td>3.2</td>
</tr>
</tbody>
</table>
3.3.3. Effect of intrinsic reactivity of different types of active sites on observed variations in activity

We have observed that both intrinsic and/or adsorbate/catalysis-induced restructuring does not lead to noticeable contributions towards observed variations in activity. This indicates that origins of temporal variation in activity between individual turnover events (dynamic variation) on the same NP and the variation in reactivity among different NPs (static variation) could be identical. It is important to classify that, when considered on a particle basis, static variation exists among individual active sites (ensembles) on the same NP, and the intrinsic reactivity of individual active sites can possibly lead to observed variations in activity over time. We believe that a possible method to deconvolute the differences between static and dynamic variations associated with observed individual catalytic turnover timescales on Au NPs is to eliminate active sites on the Au NP. This can be achieved by adding small molecule adsorbates with strong affinity to Au NPs. In addition, the process of eliminating the active sites could potentially aid in distinguishing the intrinsic reactivity amongst individual active site populations. Thiols are known to bind very strongly, and in an irreversible manner, to the surface of Au.\(^\text{44}\) In a previous study, we have confirmed this by focusing on the thermodynamic adsorption profiles of carboxylic acid-terminated thiols binding to Au NP surfaces utilizing ITC.\(^\text{45}\) Furthermore, ReaxFF MD simulations demonstrate the adsorption of a single thiol on a 1 nm Au cluster has little influence on the MSD, while the addition of two thiols decreases the restructuring behavior of Au surface atoms significantly. The addition of four thiols (\(\theta_{\text{thiol}} = 0.36\)) completely eliminates particle restructuring (Figure 3.1).
We carried out single-molecule and ensemble level measurements to measure the influence of thiols on the dispersive nature of 5.4 nm Au NPs (Figures 3.6 and 3.7 A-B). At the single-molecule level, the citrate covered Au NPs were ligand-exchanged (0-100% with respect to Au surface atoms (Auₖ) covered) with nominal amounts of thiols (C₂-TGA, C₃-MPA, C₆-MHA, and C₁₁-MUA) after they were electrostatically bound to a glass coverslip previously functionalized with alkylamine groups. A thiol with a carboxylic acid terminal group was chosen in order to ensure that the NPs were still wetted by the solution. It is important to note that the adsorption of thiols to Au leads to the formation of strong Au-S bond in a 3-fold hollow geometry.⁴⁶,⁴⁷ With increasing C₃-MPA coverage (the % of Auₖ covered is based on the 1:3 thiol:Auₖ on a 5.4 nm Au NP (the apparent reaction order in C₃-MPA is -1), the overall rate decreases linearly and saturates at approximately θₜₕᵢₒₜ = 0.3 at both ensemble and single-molecule level (Figure 3.7A-B). Our results are consistent with a separate work carried out by Nigra et al., where they determine the rate for the reduction of resazurin to resorufin on 3.5 nm Au NPs as a function of organic thiol coverage, and report that no variation in rate occurs beyond θₜₕᵢₒₜ = 0.3.⁴⁸ This reduction in reaction rate indicates that the presence of thiols leads to a reduction in the total number and type of Au active sites available for catalysis. Although the coverage-dependent trends between ensemble and single-molecule level look similar, we noticed the reaction rate at ensemble level is an order of magnitude lower than the reaction rate at the single-molecule level. We have addressed this difference in observed rates through a separate temperature-dependent single-molecule and ensemble-level study on the kinetics of resazurin reduction, wherein we concluded that, the differences in rates were due to competitive adsorption between the solvent and reactant molecules at the ensemble-level.⁴¹
Figure 3.6. The apparent first-order conversion of resazurin to resorufin in the presence of varying amounts of (A) C₃-MPA and (B) β-alanine on the Au nanoparticle surface. All experiments were carried out at 4 μM resazurin, 1mM NH₂OH, and appropriate C₃-MPA/β-alanine coverage at a constant temperature of 25 °C on 5.4 nm Au NPs.
Figure 3.7. Comparison of (A) ensemble-level and (B) single molecule level rates as a function of C₃-MPA coverage. The x-axis represents the % of Au surface atoms (Auₜ) covered by C₃-MPA with the preferred stoichiometry of C₃-MPA : Auₜ = 1 : 3. All experiments were carried out at 4 μM resazurin, 1mM NH₂OH, and appropriate C₃-MPA coverage at a constant temperature of 25 °C on 5.4 nm Au NPs. Error bars are S.D.
At the single-molecule level, we further examined the variation in rate with thiol coverage and we observed the presence of two different slopes. The <rate> decreases rapidly when the amount of Au surface atoms covered increased from 0 to 10%. An additional increase of thiol coverage from 10 – 100% resulted in a slight/no decrease in observed <rate> with a near-zero slope (Figure 3.7B). When using strongly interacting ligands such as thiols, we hypothesize that the most active under-coordinated sites will be titrated first, followed by sites with lower activity (chromatographic titration) during reaction rate measurements. This is based on the fact that the under-coordinated sites will have the strongest binding affinity for the thiol molecules.\textsuperscript{49} Previous ITC experiments of the binding of C\textsubscript{2}-TGA, C\textsubscript{3}-MPA, and C\textsubscript{6}-MHA on 5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm Au NPs showed that the differential enthalpy of adsorption (\(\Delta H_{\text{diff}}\)) decreases with increasing thiol coverage suggesting that the more active sites with increasing activity is titrated first.\textsuperscript{45} The amount of thiol used to cover 10% of the Au surface atoms resulted in ~ 30% decrease in <rate> and this indicates that the most active sites present as a fraction of total Au surface atoms is approximately 10%. From the statistics of Au surface atoms on a 5 nm Au NP (Table 3.2), the fraction of the most active under-coordinated sites (corners and edges) to the total number of Au surface atoms is ~ 10.2% (120 corner and edge atoms out of 1178 total surface atoms). The decrease in the rate for a thiol with a coverage of > 10% (the rate decreased by 50% as the % of Au\textsubscript{s} covered increased from 10 – 100%) is a result of thiol molecules binding to the Au (100) and Au (111) terrace sites of lower reactivity (1058 atoms out of 1178 total surface atoms which corresponds to 90% of the total Au surface atoms). It is important to note that we observe a reaction event at 100% thiol coverage, illustrated by a non-zero value of the reaction rate in Figure 3.7B. This is indicative of the presence of certain Au sites that cannot be blocked by
the C₃-MPA molecules and are still accessible to the reactant molecules and serve as catalytically active sites. This behavior is not observed during ensemble level measurements due to the sensitivity of the UV-Vis spectroscopy measurement (Figure 3.7A). Our observations of a decrease in <rate> and interpretations of chromatographic titrations of Au active sites of higher to lower activity are consistent with those recently reported by Nigra et al.⁴⁸

In addition, the influence of intrinsic reactivity of individual types of Au active sites on catalytic turnover events should be manifested in the values of activation and desorption energies. The procedure to determine the activation energy (Eₐ,SM) and desorption energy (E₅₉) from single-molecule measurements is described in detail in our previous work.⁴¹ We carried out temperature-dependent (20-45°C) single-molecule measurements of resazurin reduction reaction on 5.4 nm Au NPs at a constant C₃-MPA coverage to cover 10% and 25% of the Auₙ, respectively, and estimated the activation energy (Eₐ,SM) and desorption energy (E₅₉) (Figure 3.8A-B). In our previous work, we reported values of Eₐ,SM = 4.6 ± 0.4 kcal/mol and E₅₉ = 0.4 ± 0.02 kcal/mol for resazurin reduction to resorufin on a 5.4 nm Au NP in the absence of any small-molecule adsorbates.⁴¹ At 10% C₃-MPA coverage, the values of Eₐ,SM and E₅₉ are 5.4 ± 0.3 kcal/mol and 0.8 ± 0.1 kcal/mol. At 25% C₃-MPA coverage, the values Eₐ,SM and E₅₉ further increased to 5.8 ± 0.5 kcal/mol and 1.04 ± 0.1 kcal/mol, respectively. The increase in Eₐ,SM and E₅₉ with increasing C₃-MPA coverage further confirms that adding thiols chromatographically titrates Au active sites from higher reactivity to lower reactivity.
Figure 3.8. Temperature dependence of (A) $\ln k_{r,SM}$ and (B) $\ln k_d$. Solid lines in (A) are linear fits and the slopes correspond to single-molecule activation energy ($E_{a,SM}$) of 4.6 ± 0.4 kcal/mol (0% C$_3$-MPA), 5.4 ± 0.3 kcal/mol (10% C$_3$-MPA), and 5.8 ± 0.5 kcal/mol (25% C$_3$-MPA), respectively. Solid lines in (B) are linear fits and the slopes correspond to desorption energy ($E_{des}$) of 0.4 ± 0.02 kcal/mol (0% C$_3$-MPA), 0.8 ± 0.1 kcal/mol (10% C$_3$-MPA), and 1.04 ± 0.1 kcal/mol (25% C$_3$-MPA), respectively. Each data point is an average from 50 Au NPs (0% C$_3$-MPA) and 5 Au NPs (10% and 25% C$_3$-MPA) and the error bars are SD. Error bars are present in (A) and (B), but difficult to see because they are small. All experiments were carried out at saturating conditions of 4 μM resazurin and 1 mM NH$_2$OH.
To further ascertain if the observed variations in activity even in the presence of thiols is not due to intrinsic and/or adsorbate induced restructuring, we extracted the sequence of individual $\tau_{\text{off}}$ and $\tau_{\text{on}}$ values from each single turnover trajectory at varying thiol coverage, and computed their autocorrelation. For a single 5.4 nm Au NP, even at 50% C$_3$-MPA, the autocorrelation plots for both $\tau_{\text{off}}$ and $\tau_{\text{on}}$ are near zero which indicates no significant autocorrelations (Figure 3.9A-B). The lack of correlation between turnover events at complete thiol coverages suggests the absence of dynamic variation in this system, which indicates the variation in the activity observed between individual Au NP is only static in nature. A careful examination of the single-molecule $\tau_{\text{off}}$ and $\tau_{\text{on}}$ values at different thiol coverages for the reduction of resazurin at single turnover resolution over individual 5.4 nm Au NPs led to the observation of two simultaneous outcomes – an increase in individual $\tau_{\text{off}}$ and $\tau_{\text{on}}$ values followed by reduced variation in activity, i.e., the catalytic turnover events transformed from being completely stochastic to being “less stochastic” in nature (Figure 3.10A). The first outcome of thiol coverage is an increase in both $\tau_{\text{off}}$ and $\tau_{\text{on}}$ due to the chromatographic elimination of catalytic sites from higher to lower activity leading to reduced variation in activity (Figure 3.10A). As the thiol coverage increases, only the active sites that have a similar lower activity exists for catalyzing the conversion of resazurin to resorufin. In the study by Nigra et al., they have attributed the non-zero catalytic activity of Au NPs at higher thiol coverages to a rise in the fraction of pinhole sites, which remain accessible to the organic thiol ligands during kinetic poisoning experiments.$^{48}$ Previous literature states that these pinhole sites can be directly related to vacancies in the self-assembled monolayer (SAM).$^{50}$ The observation of $\tau_{\text{off}}$ and $\tau_{\text{on}}$, even when 100% of the Au surface sites are covered by C$_3$-MPA, is most likely attributed to the presence of vacancies in
the SAM. However, we did not observe any reaction events at coverages > 50% for higher alkyl chain length thiols (C$_6$-MHA and C$_{11}$-MUA). We believe the reason for this behavior is that higher chain length thiols form highly ordered SAMs on the surface of Au NPs when compared to lower chain length thiols, thereby reducing the vacancies present in the SAM.
Figure 3.9. (A, B) ACF of at (A) $\tau_{off}$ and (B) $\tau_{on}$ at 25% C3-MPA determined from the same catalytic turnover trajectory on a single 5.4 nm Au NP. It indicates successive catalytic turnover events are uncorrelated (one would expect events on different active sites to be uncorrelated), supporting the absence of dynamic variation even at increasing thiol coverage. All experiments are carried out at 4 $\mu$M resazurin and 1 mM NH$_2$OH. Each distribution was collected from a single turnover trajectory on a 5.4 nm Au nanoparticle with ~ 500 turnover events recorded.
Figure 3.10. (A) A segment of the fluorescent trajectory with increasing C$_3$-MPA coverage on the 5.4 nm Au NP surface. The percentages indicate the % of Au surface atoms covered by C$_3$-MPA. The result demonstrate the individual waiting times increase to a much greater extent with increasing C$_3$-MPA coverage leading to chromatographic elimination of catalytic sites from higher to lower activity. (B) The dependence of $<\tau_{off}>$ (top) and $<\tau_{on}>$ (bottom) on alkanethiol chain length at 10% (■) and 25% (●) thiol coverage. Error bars correspond to the S.D. values. The values of $<\tau_{off}>$ and $<\tau_{on}>$ for C$_2$-TGA and C$_3$-MPA are similar and that represents the kink in the data (otherwise linear) with increasing thiol alkyl chain length (C$_2$ – C$_{11}$) indicating an increase in the waiting time between individual catalytic turnovers. Reaction conditions were 4 µM resazurin, 1mM NH$_2$OH, 298 K, and appropriate thiol coverage. Each distribution was collected from a single turnover trajectory on a 5.4 nm Au nanoparticle with ~ 500 turnover events recorded.
The increase in the value of $<\tau_{off}>$ with increasing thiol coverage can be addressed by looking more carefully at the individual contributions to an observed $\tau_{off}$ value during each catalytic turnover event. The waiting time before product formation ($\tau_{off}$) has contributions from the time taken for diffusion through the boundary layer to the particle surface ($\tau_{diff}$), the time taken for adsorption ($\tau_{ads}$), and the time taken for reaction ($\tau_{rxn}$). By assuming the Au NP surface (without the presence of thiols) to be a flat plate with regards to the reactant molecule, we determined the time taken for diffusion through the boundary layer ($\tau_{diff}$) to be $\sim 10^{-8}$ s. The value of $\tau_{diff}$ remains fairly constant even in the presence of thiols on the Au NP surface. Coupled with the fact that the reaction event is instantaneous, the values of $\tau_{rxn}$ and $\tau_{diff}$ should not contribute significantly to the observed values of $\tau_{off}$. The contribution of $\tau_{ads}$ to the observed values of $\tau_{off}$ can be ascertained by determining the rate of adsorption ($R_{ads}$) of resazurin molecule to the Au surface (see Appendix 3.6.1). The $R_{ads}$ is related to the collision frequency of resazurin to the Au NP surface ($J_s$) and the sticking probability ($S$) ($R_{ads} = J_s \times S$). Jung et al. determined the initial sticking probabilities ($S_0$) and sticking probabilities ($S$) of alkanethiols of various chain lengths from ethanol onto Au as a function of coverage and identified that the value of $S$ decreased linearly with coverage. We determined the value of $J_s$ for resazurin molecules to the Au surface ($J_s = 0.16$ moles/m$^2\cdot$s). By assuming the value of $S_0$ for a C$_3$-thiol (as the length of a C$_3$-thiol of $\sim 0.6$ nm and can be compared to the length of $\sim 0.5$ nm of a resazurin molecule) from Jung et al., we determined the value of $R_{ads}$ of resazurin molecules to the surface. This gives an upper limit on the value of $R_{ads}$ as the resazurin molecules will possess a value of $S$ that is lower than the value of $S$ for thiols (see SI for detailed calculations). The inverse of $R_{ads}$ gives the value of $\tau_{ads}$. At 100% thiol coverage, the lower limit value of $\tau_{ads}$ for resazurin is determined to be $\sim 8$
s (by assumption of the value of \(S_0\) for C3-thiol to represent the value of \(S\) for resazurin). This assumption is valid as the surface is completely covered with thiols and the resazurin molecules most likely adsorb with a value equal to its initial sticking probability (\(S_0\)). These timescales observed for \(\tau_{ads}\) are in the same order as the timescales observed for \(\tau_{off}\). Although this determination of \(R_{ads}\) and \(\tau_{ads}\) is an approximate estimation, it indicates the dominant contribution of \(\tau_{ads}\) to the observed values of \(\tau_{off}\) in the presence of thiols. In addition, the waiting times increase linearly as a function of alkyl chain length of the thiol (Figure 3.10B). This could be manifested through a decreased resazurin adsorption probability coupled with the fact that higher alkyl chain length thiol forms a more ordered SAM, which would influence \(\tau_{off}\), or an additional barrier to desorption, which would influence \(\tau_{on}\).

The second outcome of reduced variations in activity is better represented by analyzing the distributions of \(\tau_{off}\) and \(\tau_{on}\) from a single 5.4 nm Au NP trajectory (Figures 3.11A-D and 3.12A-C). At 0% C3-MPA, the distributions of \(\tau_{off}\) follow a single exponential decay which can be correlated to a multitude of sites available on the Au NP to catalyze the conversion of resazurin to resorufin. Currently, we cannot resolve whether the turnover is occurring on the same site, an adjacent site or a site many molecular distances away from the previous turnover (Figure 3.11A). As the coverage of C3-MPA increases, we “modulate” the surface such that we reduce the absolute number of active sites. This indicates that the single molecule turnover events correspond to a distribution of \(\tau_{off}\) that approximate a Gaussian distribution (Figure 3.11B-D). Evolution of the single exponential decay to a Gaussian distribution suggests two possibilities: (i) each individual catalytic turnover event occurs on the same type of active sites or (ii) more than one process occurs on the single Au NP in the presence of increasing thiol coverage. The thiol molecules are present only to kinetically
block the Au active sites and it is unlikely that the presence of thiols would result in additional process during the reduction of resazurin to resorufin. Thus, with increasing thiol coverages, the presence of a Gaussian distribution can be attributed to the catalytic turnover events occurring on the same type of Au active sites with lower intrinsic reactivity.
Figure 3.11. (A-D) Distributions of $\tau_{off}$ from a single 5.4 nm Au NP trajectory at 25 °C covered with (A) 0%, (B) 10%, (C) 25%, and (D) 50% C$_3$-MPA. Solid line in (A) is a single-exponential fit and in (B-D) solid lines are Gaussian fits. Reaction conditions were 4 µM resazurin, 1mM NH$_2$OH, 298 K, and appropriate thiol coverage. Each distribution was collected from a single turnover trajectory on a 5.4 nm Au nanoparticle with ~ 500 turnover events recorded. Bin widths (W) were calculated using the standard published formula ($W = 3.49\sigma N^{-1/3}$, where $N$ – number of observation and $\sigma$ is estimate of the standard deviation).\textsuperscript{51}
Figure 3.12. Distributions of $\tau_{on}$ from a single 5.4 nm Au NP trajectory at 25 °C covered with (A) 10%, (B) 25%, and (C) 50% C$_3$-MPA. Solid lines in (C-E) are Gaussian fits. All experiments were carried out at 4 μM resazurin, 1 mM NH$_2$OH, and appropriate C$_3$-MPA coverage. Each distribution was collected from a single turnover trajectory on a 5.4 nm Au nanoparticle with ~ 500 turnover events. Bin widths (W) were calculated using the standard published formula ($W = 3.49\sigma N^{-1/3}$, where N – number of observation and $\sigma$ is estimate of the standard deviation).\textsuperscript{51}
It is well known that changing adsorbate-surface interactions leads to variations in activity for metal NP and bulk metals in heterogeneous catalysis.\textsuperscript{14,52} To quantify the static variation prevalent in the thiol-covered Au NPs, we calculated the mean value of the turnover rate and its relative standard deviation (RSD) over each particle by considering C\textsubscript{11}-MUA. C\textsubscript{11}-MUA was chosen because it is known to form a highly ordered SAM on the Au surface and represent the longest alkyl chain length thiols we have used in our experiments. Based on the statistical analysis of 10 different 5.4 nm Au NPs (each responsible for \(\sim\) 500 turnover events) (Figure 3.13), the 0\% covered Au NPs exhibited higher activity (\(<\text{rate}>\), 0.28 – 0.52 s\(^{-1}\)) and greater variation in activity (RSD, 44 – 87\%) for the resazurin reduction reaction. However, the 50\% C\textsubscript{11}-MUA covered Au NPs exhibited extremely reduced activity (\(<\text{rate}>\), 0.04 – 0.06 s\(^{-1}\)) and variation in activity (RSD, 7 – 10\%). Overall, the single-molecule measurements in the presence of thiols indicate that the origins of variations in activity among individual turnover events are related to the intrinsic reactivity of individual types of Au active sites on an Au NP and can effectively be modulated to a great extent by controlling the absolute number of active sites catalyzing the reaction.
Figure 3.13. Distribution of mean value of turnover rate and its relative standard deviation (RSD) over 10 different 5.4 nm Au NPs for (●) 0%, (●) 10%, (●) 25%, and (●) 50% C_{11}-MUA. All experiments are carried out at 4 μM resazurin, 1 mM NH_{2}O, and 25°C. Each distribution was collected from a single turnover trajectory on a 5.4 nm Au nanoparticle with ~500 turnover events recorded.
To confirm the adsorbed thiol on the Au NP is responsible for the reduction in the number of active sites and the observed difference in waiting times, we repeated the single-molecule experiments utilizing an adsorbate that binds weakly to gold. The adsorption of β-alanine (a structure similar to C₃-MPA, where in the thiol group is replaced by an amine) was measured by ITC and had an adsorption affinity of $10^3$ M$^{-1}$: four orders of magnitude lower than C₃-MPA. ITC measurements demonstrate both resazurin and resorufin bind to solvated Au NPs with an affinity and enthalpy comparable to β-alanine. The single molecule trajectories in the presence of different coverages of β-alanine looked identical to the NP trajectory in the absence of any small molecule adsorbate (Figure 3.14A). In addition, the distribution of $\tau_{off}$ and $\tau_{on}$ from the turnover trajectory of one 5.4 nm Au NP at a saturating substrate concentration (4 μM) and 50% β-alanine coverage follows a single-exponential decay (Figure 3.14B-C). This suggests that the dynamics of catalytic turnovers in the presence of β-alanine is similar to the Au NP without any adsorbate and completely different from the thiol-covered Au NPs.
Figure 3.14. (A) A segment of the fluorescent trajectory with increasing β-alanine coverage on the 5.4 nm Au NP surface. The percentages indicate the % of Au surface atoms covered by β-alanine. It is important to note that these %s are theoretical values as β-alanine does not adsorb to the Au surface. The result demonstrate the individual waiting times are similar increasing β-alanine coverage indicating that β-alanine does not modulate kinetic dispersion of Au NPs. (B,C) Distributions of (B) $\tau_{\text{off}}$ and (C) $\tau_{\text{on}}$ from a single 5.4 nm Au NP trajectory at 25 °C covered with 50% β-alanine. Solid line in (B,C) is a single-exponential fit. All experiments were carried out at 4 μM resazurin, 1mM NH$_2$OH, and appropriate theoretical coverage of β-alanine. Each distribution was collected from a single turnover trajectory on a 5.4 nm Au nanoparticle with ~ 500 turnover events recorded.
3.4. Conclusions

The variations in activity and the heterogeneous nature of individual Au NPs have been elucidated and modulated using carboxylic acid-terminated thiols of varying alkyl chain length along with utilizing the reduction of resazurin to resorufin as a model reaction. Through ReaxFF MD simulations and single-molecule kinetic measurements, we have confirmed that neither intrinsic nor adsorbate/catalysis-induced restructuring lead to the observed variations in activity among individual catalytic turnover events. Comparison with geometrical calculations in the literature led to the identification of different sites as edges, corners, and surface sites. The intrinsic reactivity of the different types of active sites and its influence on the observed variation in activity was probed by adding thiols of varying amounts. The increase in coverage of thiols led to a chromatographic titration of the Au active sites from higher to lower activity. In addition, the evolution of a single exponential decay to a Gaussian distribution suggested that the successive catalytic turnover events tend to occur on the same type of active site with lower reactivity. By coupling single-molecule approach with the ability of small molecule adsorbates to titrate Au active sites, this study reveals that the origins of variations in the activity in nanoparticle catalysts is strongly dependent on the intrinsic reactivity of different types of active sites. This study offers a fundamental understanding of controlling catalytic activity through kinetically blocking active sites and provides insight into unique avenues that can be probed using single-molecule level catalytic experiments. The ability to control the number of active sites on a NP surface has strong implications in the study of heterogeneous catalysis; more specifically where metal NPs are employed as catalysts.
3.5. References


3.6. Appendix

3.6.1. Determination of collision frequency and rate of adsorption of resazurin molecule to Au NP surface

The rate of adsorption of a molecule \( R_{ads} \) to a surface is defined as the product of collision frequency \( J_S \) of a liquid-phase solute to the surface and the sticking probability \( S \).\(^{44}\)

\[
R_{ads} = J_S \times S
\]  \hspace{1cm} (S1)

The sticking probability is defined as the probability that the molecule will adsorb upon its collision with the surface.\(^{44}\) The collision frequency is given by:

\[
J_S = C_S \times \sqrt{\frac{K_B T}{2\pi m}}
\]  \hspace{1cm} (S2)

where, \( C_S \) is the concentration of the solute in that liquid nearest to the surface, \( K_B \) is the Boltzmann constant, \( T \) is the temperature, and \( m \) is the mass of the adsorbing solute.

We have carried out our single-molecule measurements on 5.4 nm Au NPs at 4 µM resazurin concentration and at a temperature of 298.15 K. The individual parameters for determining the collision frequency of resazurin molecules to the Au surface is calculated as follows:

Concentration of resazurin in water nearest to the Au surface \( (C_S) = 4 \text{ µM} = 0.004 \text{ moles/m}^3 \)

Boltzmann constant \( (K_B) \)
\[
= 1.38 \times 10^{-23} \text{ m}^2\cdot\text{kg/s}^2\cdot\text{K}
\]

Temperature \( (T) \)
\[
= 298.15 \text{ K}
\]
Molecular weight of resazurin ($M_w$) = 229.19 g/mol

Mass of resazurin ($m$) = $3.81 \times 10^{-25}$ kg/molecule

Collision frequency of resazurin to the surface ($J_S$) = 0.166 moles/m$^2$·s

Diameter of the nanoparticle used ($d$) = 5.4 nm

Surface area of the 5.4 nm Au nanoparticle = $7.85 \times 10^{-17}$ m$^2$

Collision frequency of resazurin as a function of time ($J_S \times A$) = $1.3 \times 10^{-17}$ moles/s = $7.9 \times 10^6$ molecules/s

Jung et al. determined the initial sticking probabilities ($S_0$) and sticking probabilities ($S$) of alkanethiols of various chain lengths from ethanol onto Au as a function of coverage and identified that the value of $S$ decreased linearly with coverage.$^{44}$ The value of $S_0$ for a C$_3$-thiol from ethanol solution onto Au surface at 298 K is reported to be $1.7 \times 10^{-8}$. At 100% thiol coverage, since the Au surface is completely covered by thiols, the sticking probability of resazurin ($S$) can be assumed to be its initial sticking probability value ($S_0$). Moreover, since resazurin is a very weak adsorbate when compared to thiols on the Au surface, the assumption of $S_0$ for a C$_3$-thiol to be the value of $S_0$ for resazurin would result in the upper limit of the value of $R_{ads}$.

Rate of adsorption of resazurin molecules to the Au surface ($R_{ads}$) = 0.133 molecules/s

Inverse of the value of $R_{ads}$ will result in the value of time taken for adsorption ($\tau_{ads}$) of resazurin molecule to the Au surface. This value will indicate the lower limit of $\tau_{ads}$. The
value of $\tau_{ads}$ is 7.9 s and this indicates its contribution to the observed longer values of $\tau_{off}$ at higher thiol coverages.
Chapter 4

Catalysis on Individual Gold Nanoparticles Reveals Reorientation

Dynamics of Single Fluorescent Product Molecules

4.1. Introduction

One of the interesting aspects of single-molecule measurements is the ability to measure the structural and conformational dynamics of an individual molecule while it interacts with bulk ligands or takes part in a catalytic cycle. In this chapter, we focus on detecting and understanding the orientation and rotational motion of individual fluorescent product molecules of a catalytic reaction on the surface of a metal nanoparticle using changes in fluorescence intensity.

The application of fluorescence microscopy to the field of single molecule analysis has allowed access to a wide range of phenomena that are obscured in ensemble measurements. The properties of fluorescent single molecules such as fluorescence lifetime, fluorescence intensity, and spectral shape and position can fluctuate in time indicating changes in the conformation of the fluorescent molecule or in the chemical or physical environment it resides. Small molecule fluorescent probes are of paramount importance for analytical applications because of the high sensitivity of fluorescence detection. The ability to monitor molecular orientation has significant impact in the field of biology, physics, material science, and chemistry. In biology, single molecule measurements have provided the medium to observe protein folding and the macromolecular motion of proteins in cell membranes. In physics, fluorescent dye molecules have been incorporated to understand the quenching and enhancement effects near a metal surface as a function of
molecular orientation. In material science, monitoring single molecule orientation is used to elucidate molecular scale motions and site heterogeneity in polymeric and self-assembled systems. In chemistry, studies on catalysis and binding that use fluorescence resonance energy transfer (FRET) as a proximity probe because the FRET efficiency depends on the relative orientations of donor and acceptor chromophores. The rotational mobility of a fluorescent molecule is expected to be dependent on its chemical and physical environment. One might expect that adsorbed fluorescent molecules on a surface (glass, polymer, or enzyme) would not possess any rotational dynamics since their interaction with the surface would presumably hold them in place. Instead, individual dye molecules physisorbed to glass and embedded in thin polymer films demonstrate a wide range of orientation dynamics which can be used as sensitive probes of their immediate environment.

Studies on orientations of fluorescent molecules near a metal nanoparticle surface leading to fluorescence quenching or fluorescence enhancement, has recently gained significant interest in a wide range of applications in material science, catalysis, biology, and photonics. When located in close proximity to a metallic nanoparticle, a fluorescent molecule exhibits strong changes in its optical and electronic properties. The presence of a fluorescent molecule on a metal nanoparticle results in quenching of its fluorescence; numerous reports on the effects of gold colloids on fluorescent molecules indicate that the emission of fluorophores positioned less than 5 nm from the Au nanoparticles (up to 30 nm in diameter) are quenched due to nonradiative energy transfer from the excited states of the fluorescent molecules to the gold nanoparticles. At distances greater than 5 nm from the metal surface, fluorescence enhancement has been observed. The quenching or enhancement effects on the orientation of the fluorescent molecule are expected to depend on...
critically on the size and shape of the nanoparticle, the location of the fluorescent molecule with respect to the nanoparticle, the orientation of the dipole moment of the fluorescent molecule with respect to the nanoparticle surface, and the overlap of the fluorescent molecule’s emission spectrum with the nanoparticle’s absorption spectrum.\cite{10} The excited fluorescent molecules in the vicinity of a metal surface are closely involved in most of the dynamic processes, and effects of their orientation on quenching/enhancement effects are subjects of strong interest. The orientation dynamics of fluorescent molecules in close proximity to a metal nanoparticle surface have been investigated at the ensemble-level.\cite{10,17,18} Ensemble-level measurements results in averaged quantities which mask the specific interactions occurring at the metal-fluorescent molecule interface so that a detailed account of the orientation dynamics of the fluorescent molecule cannot be determined.\cite{26,27} The orientation and rotational dynamics of fluorescent molecules of interest can be understood by focusing on one molecule at a time.

In the present chapter, we utilize total internal reflection fluorescence (TIRF) microscopy at the limit of single-molecule detection to study the orientational/rotational dynamics of a single fluorescent molecule (resorufin) generated on the surface of a 5.4 nm Au nanoparticle as a result of the reaction between resazurin (non-fluorescent) and hydroxylamine. Through careful analysis of fluorescence intensity versus time traces for each catalytic turnover event, we have identified the presence of a slow rise in the fluorescence intensity for the observed on-time (rise time) for certain catalytic turnover events. We attribute the presence of rise times to the reorientation dynamics of resorufin on the Au surface. Through theoretical calculations, we have identified the most preferred adsorption configuration of resorufin on Au (111) surface and have determined the rate at which a
resorufin molecule emits fluorescence as a function of molecular angle of rotation with respect to the Au surface. We also performed ensemble level stopped-flow measurements on the same system and identified the presence of a delay time before the fluorescence emission of resorufin, an observation that we attribute to rotational dynamics of resorufin on the Au surface. We aim to relate experimental observations of rise times to the theoretical predictions of reorientation dynamics of resorufin.

4.2. Experimental

4.2.1. Materials

Resazurin, resorufin, and hydroxylamine (NH$_2$OH) were obtained from Sigma Aldrich (Milwaukee, WI). An aqueous dispersion of gold nanoparticles (5.4 ± 0.7 nm diameter) stabilized by citrate were obtained from Ted Pella (Redding, CA). Milli-Q grade water (18.2 MΩ·cm) was used in all experiments. All chemicals were used as received.

4.2.2. Single molecule measurements using TIRF microscopy

Single-molecule experiments were performed on an Olympus IX-71 total internal reflection fluorescence (TIRF) microscope. The microscope is illuminated using a CW Argon/Krypton-ion laser (35-KAP-431-220, Melles Griot, Carlsbad, California, USA) with 476 to 676 nm excitation; output power 4 to 20 mW. A continuous wave polarized 520 nm beam was focused on the sample for the fluorescence excitation of resorufin. An oil immersion objective (PlanAPO 60×/1.45 NA, TIRFM-2, WD 0.15 mm) was used for collection of the fluorescent photons. For TIRF, laser light is coupled via a fiber-optic cable to the TIRF illuminator on one end of the epiport and focused off-center on the back aperture of the objective. A Peltier-cooled 12-bit digital CCD camera (SensicamQE) with high
resolution, sensitivity, and high quantum efficiency (65%) in the visible range was used to
detect photons emitted by resorufin. The camera was modified with a fast shutter to enable
the collection of two successive images with an interval of 500 ns. The camera was operated
at 45 msec time resolution for all the single-molecule experiments. Movies of fluorescence
bursts were analyzed using a home-written Mathematica program (Appendix A), which
extracts the individual fluorescence intensity trajectories from localized fluorescent spots
over the entire duration of the movie. The obtained individual fluorescence trajectories were
further analyzed using a home-written Mathematica code (Appendix B), which determines
rise times associated with each individual trajectory and calculates the distribution of the
obtained rise times.

A 40 mm glass cover slip is functionalized with 3-aminopropyltriethoxysilane
(APTES) and citrate-stabilized 5.4 nm Au nanoparticles are electrostatically-adsorbed onto
this surface. The cover slip is incorporated into a perfusable fluid optical cavity (that includes
a microaqueduct slide) which is fixed on the stage of the microscope. The top surface of the
microaqueduct slide is coated with an electrically conductive transparent film of Indium-Tin
oxide (ITO) and is used to remove heat from the optical cavity. Temperature control is
provided by circulating a coolant fluid through an O-ring sealed window cooling adapter
placed on the surface of the microaqueduct slide. The Au nanoparticles are separated from
the cooling fluid and are in contact only with the reactant solution. The temperature is
measured by placing a Type-T thermocouple (Omega Engineering Inc.) on the surface of the
microaqueduct slide.28

4.2.3. Ensemble-level measurements using stopped-flow spectroscopy
Ensemble-level stopped-flow measurements were carried out using a SF-300X stopped-flow spectrometer (KinTek Corporation, Austin, TX) at 1 msec time resolution (the time resolution is determined by the mixing time which is equal to 1 msec). The instrument consists of a high intensity, stable arc lamp source (L2274 Xenon, Hamamatsu Corporation) with 200 – 800 nm excitation that illuminates the sample using a parabolic mirror and lens-free optics to provide optimal throughput to a monochromator. The arc lamp requires circulating water bath for operation. The water in the supplied bath was not cooler than room temperature to prevent condensation inside the light source housing. A continuous wave 570 nm beam was focused on the sample for the fluorescence excitation of resorufin. The fluorescent molecule, resorufin, absorbs at 570 nm and emits photons at 581 nm. A long pass filter (575 nm, 25.4 mm OD, Edmund Optics) is placed inside a filter slider and is used to collect the fluorescence emission of resorufin. The stopped-flow instrument consists of a photo-multiplier tube (PMT) detector and a photodiode and thus provides the ability to detect and record the fluorescence emission and absorbance of resorufin simultaneously in a single experiment. The reactant samples are loaded into a syringe chamber through a pair of syringes (BD 3 ml Luer-Lok™ tip). The reactant sample (resazurin/NH₂OH) was filled in one of the syringes and the Au NP solution was filled in the other syringe. The instrument consists of a unique two-position valve to allow easy and efficient sample loading. In the “LOAD” position, the two syringes are connected to their individual sample load ports. In the “FIRE” position, the two syringes are driven to initiate the mixing of reactants into the observation cell. Optical sensors read the position of the sample load valve and inform the computer whether the valve is in the appropriate position for loading or firing. The observation cell can hold up to 40 μL of the reactant and thus each “shot” consists of 20 μL
of each reactant mixed in the observation cell. The reactants are rapidly mixed in the observation cell and fluorescence emission and absorbance of resorufin is detected simultaneously for a time period of 1 s. A total of 15-20 kinetic traces of 1 s duration were routinely averaged for each experiment. Temperature control is achieved by using a circulating water bath. Water circulates through the stainless steel cube surrounding the observation cell, then flows through the syringe chamber and exits from the top of the syringe chamber. A solid-state temperature sensor is located in the syringe chamber, providing a continuous readout to the computer which can then be used to calibrate the results to the real-time temperature. The KinTek SF-300X is computer controlled; this synchronizes the instrument’s drive system with the detector gain and ensures the sample load valve is in the correct position. The emission and absorbance data were analyzed using the KinTek Global Kinetic Explorer (KinTek Corporation) software.

4.2.4. Density Functional Theory (DFT) calculations to determine preferred orientation of resorufin on Au surface and resorufin binding energy as a function of molecular angle

All DFT calculations were carried out using the Vienna ab initio simulation program (VASP); an ab initio total energy and Molecular Dynamics program developed at the Institute for Material Physics at the University of Vienna. The Perdew-Wang (PW91) version of the generalized gradient approximation (GGA) was used with a plane wave basis set cutoff energy of 450 eV and a 3×3×1 Monkhorst-Pack k-point mesh. Structural optimization was carried out until the forces on all atoms were less than 0.02 eV/Å. Spin polarized total energy calculations were employed for all surface analyses. All adsorbed species were placed on the fcc (111) surface of a 4×4 slab of gold four layers thick with the bottom two layers frozen, and using 14 Å of vacuum. Calculations were also performed with
24 Å of vacuum and yielded values within 0.1 eV of those using 14 Å of vacuum. The DFT-D2 method of Grimme,$^{35}$ as implemented in VASP, was used to account for dispersion interactions. This adds an empirical dispersion term to the calculated DFT energy that is the sum of all atomic pair interactions in the system as described by Grimme$^{35}$:

$$E_{\text{disp}} = -s_6 \sum_{l=1}^{N_{\text{at}}-1} \sum_{j=l+1}^{N_{\text{at}}} \frac{C_6^{ij}}{R_{ij}^6} f_{\text{damp}}(R_{ij})$$

(1)

Where $E_{\text{disp}}$ is an empirical dispersion correction, $N_{\text{at}}$ is the number of atoms in the system, $C_6^{ij}$ denotes the dispersion coefficient for atom pair $ij$, $s_6$ is a global scaling factor that only depends on the standard density functional used, and $R_{ij}$ is an interatomic distance. In order to avoid near-singularities for small $R$, a damping function $f_{\text{damp}}$ must be used. Since gold is not included in the list of elements published by Grimme, the $C_6$ and vdW radius were assigned the values of 40.62 J nm$^6$ mol$^{-1}$ and 1.772 Å, respectively.$^{36}$ The $s_6$ global scaling factor was set to 0.75, which is the accepted value for the PBE functional and has been shown to correlate well with the PW91 functional.$^{35,37}$

### 4.3. Results and Discussion

#### 4.3.1. Determination of rise times through single molecule conversion of resazurin to resorufin

In Chapter 2, we tracked individual catalytic turnover events at different temperatures on single 5.4 nm Au NPs by monitoring the reduction of resazurin to resorufin by NH$_2$OH in real-time. By maintaining a constant flow of resazurin (4 μM) and NH$_2$OH (1 mM), we measured individual fluorescent bursts due to the production of a single molecule of resorufin on the surface of a 5.4 nm Au NP in the TIRF microscope interfaced with a
temperature controlled cell. The individual turnovers are represented by two waiting times, $\tau_{\text{off}}$ and $\tau_{\text{on}}$. A careful observation of each individual fluorescence turnover event indicated the presence of a slow rise in fluorescence intensity to the observed on-time for certain catalytic turnover events (Figure 4.1A), which is the rise time ($\tau_{rt}$). On average, out of ~1000 individual catalytic turnover events in a single fluorescence trajectory, the % of turnover events which had an associated rise time amounts to 20-25%. We utilized a home-written Mathematica code to extract $\tau_{rt}$ for each individual turnover event in an entire fluorescence trajectory. The average of these rise times $<\tau_{rt}>$ was computed by lining up each individual turnover event on top of each other on its left edge and determining an average turnover off-on event (Figure 4.1B).
Figure 4.1. (A) A segment of a single molecule fluorescent trajectory showing the presence of rise times (red circles). (B) Representation of $\tau_{rt}$ and $\tau_{on}$ associated with a catalytic turnover of resazurin to resorufin. All experiments are carried out in 4 μM resazurin and 1 mM NH$_2$OH.
We have observed the presence of rise times in all our temperature-dependent single molecule data (5 - 45°C). This observation of $\tau_{rt}$ can possibly indicate the presence of a reorientation event associated with the fluorescent resorufin molecule which occurs immediately after the product formation event (reaction) and during the fluorescence emission event (observed as the intensity increase in the off-on fluorescence trajectory). One would argue that the reaction and photon emission from the fluorescent product molecule is spontaneous and should be observed as instantaneous intensity jumps within the 45 msec time resolution used in our experiments. However, a study by Weston et al.\textsuperscript{13} on individual DiIC\textsubscript{18} molecules physisorbed to glass and embedded in thin, spin-cast polymer films revealed that reorientation dynamics of individual dye molecules can be measured with confocal microscopy with a 32 msec time resolution. They identified a fraction of the total fluorescent dye molecules observed were susceptible to rotational dynamics and reported that dye molecules reorient with a higher frequency on glass and thinner polymer films. From Figure 4.1A-B, the relationship between $\tau_{off}$ and $\tau_{rt}$ can be ascertained. We mentioned in Chapters 2 and 3 that $\tau_{off}$ represents the combined waiting time of three possible phenomena: the time taken for diffusion ($\tau_{diff}$), the time taken for adsorption ($\tau_{ads}$), and the time taken for reaction ($\tau_{rxn}$). We believe that $\tau_{diff}$ and $\tau_{ads}$ is associated with the diffusion and adsorption of non-fluorescent resazurin molecule while $\tau_{rxn}$, which happens in the picosecond timescale, is part of the observed $\tau_{rt}$. In addition to the contribution from $\tau_{rxn}$, the observed $\tau_{rt}$ can possibly include contributions associated with molecular rotation of the resorufin molecule on the Au surface and its accompanying emission dynamics. Here, emission dynamics can refer to the fluorescence enhancement/quenching effects of the fluorescent resazurin molecule when it is present in close proximity to the Au nanoparticle surface. Although, these three events that
can contribute towards the observed $\tau_{rt}$ may possibly occur at different timescales, it is impossible to deconvolute their individual contributions to the observed $\tau_{rt}$ with our 45 msec time resolution. Overall, the waiting time ($\tau_{off}$) before the product formation can be written in terms of its individual contributors.

\[ \tau_{off} = \tau_{dark} + \tau_{rt} \]  
\[ \tau_{dark} = \tau_{diff} + \tau_{ads} \]  
\[ \tau_{rt} = \tau_{rxn} + \tau_{molecular\ rotation} + \tau_{emission\ dynamics} \]

In Chapter 2, we measured the kinetics of resazurin reduction to resorufin and determined the rate of product formation ($<\tau_{offs}>^{-1}$) as a function of resazurin concentration. At each resazurin concentration, we determined the average value of rise times $<\tau_{rt}>$ from the time versus fluorescence intensity data. The inverse of the $<\tau_{rt}>$ gives the rate of product reorientation ($<\tau_{rt}>^{-1}$) as a function of resazurin concentration (Figure 4.2). The value of $<\tau_{rt}>^{-1}$ was found to be independent of resazurin concentration, which is acceptable since we would expect the value of $\tau_{rxn}$ to be independent of resazurin concentration. Additionally, since we perform our single-molecule measurements with single turnover resolution, i.e., we observe the formation of one product molecule at any given time, the rotational dynamics associated with each resorufin molecule may also be unperturbed by the presence of a resazurin molecule. This would account for the observed constant values of $<\tau_{rt}>$ as a function of resazurin concentration.
Figure 4.2. Resazurin concentration dependence of $\tau_{\text{off}}^{-1}$ and $\tau_r^{-1}$ at 25°C. Each data point is calculated from ~500 individual turnover events. All experiments are in 1 mM NH$_2$OH. Solid red lines are fits of the corresponding rate expressions for $\tau_{\text{off}}^{-1}$ provided in Chapter 2. The data for $\tau_{\text{off}}^{-1}$ is reproduced from Chapter 2. All error bars in the graphs are a result of the average values from 10 Au NPs.
4.3.2. Differentiation between timescales associated with reactant diffusion and adsorption ($\tau_{\text{dark}}$) and timescales associated with reaction and product reorientation ($\tau_{\text{rt}}$)

A turnover time trajectory contains valuable information regarding temporal correlations between turnovers which are inaccessible to ensemble-level data. Unlike enzymes, where one can evaluate differential reactivity among individual enzymes (static dispersion) and the reactivity of the same enzyme over time (dynamic dispersion), the dispersion of nanoparticles is predominantly static in nature as we cannot differentiate between individual active-sites on a given nanoparticle. From Chapter 3, we show the intrinsic reactivity of different types of active sites on the Au nanoparticles was determined to be responsible for the observed variations in activity between individual turnover events on the same Au nanoparticle. Furthermore, we performed an autocorrelation analysis on the observed $\tau_{\text{off}}$ and $\tau_{\text{on}}$, and identified that waiting times between individual turnover events is completely uncorrelated. With the identification of rise times being prevalent for only certain catalytic turnover events, an autocorrelation analysis on the observed rise times is warranted. Moreover, the original value of each $\tau_{\text{off}}$ is also represented as the sum of two contributions ($\tau_{\text{dark}} + \tau_{\text{rt}}$). To confirm the contributions of $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ to the observed variations in activity, we extracted a sequence of individual $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ values from each turnover trajectory, and then calculated their autocorrelation function (the formula and the procedure for determining the autocorrelation function is mentioned in Chapter 3). For one Au nanoparticle at the saturation resazurin concentration (4 μM at 25°C), the autocorrelation function for $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ revealed no significant correlations between individual catalytic turnover events, i.e., all individual values of $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ were completely uncorrelated and were found to lie within the 95% CI values (Figure 4.3A-B). We repeated this procedure for data at 5, 10, 20, 35, and
45°C and observed that $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ between individual turnover events are completely uncorrelated. This result confirms that both reactant diffusion + adsorption event and reaction + product reorientation dynamics are not responsible for observed activity fluctuations and falls in line with the results from Chapter 3 that temporal variations in activity on a single Au nanoparticle is mainly related to the intrinsic reactivity of different types of sites.

Since $\tau_{\text{rt}}$ is observed only for certain catalytic turnover events, it is imperative to understand the relationship between the observed $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ from the same catalytic turnover event, i.e, whether a long $\tau_{\text{dark}}$ is followed by a long $\tau_{\text{rt}}$ and vice-versa. The distinction between $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ is further determined by the correlations between the observed $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ values from each individual turnover event from the same fluorescence trajectory. The correlation coefficient $\rho_{x,y}$ between two variables $x, y$ is defined as,

$$\rho_{x,y} = \frac{\langle (x y) - \langle x \rangle \langle y \rangle \rangle}{\sqrt{\langle (x^2) \rangle \langle (y^2) \rangle}}$$

where $<>$ denotes averaging. The value of $\rho_{x,y}$ is between -1 and 1: if $x$ and $y$ are completely correlated, $\rho_{x,y} = 1$; if completely uncorrelated, $\rho_{x,y} = 0$; and if completely anticorrelated, $\rho_{x,y} = -1$. No significant correlation is observed between $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ for individual Au nanoparticles at any given temperature, with their correlation coefficient $\rho_{\tau_{\text{rt}},\tau_{\text{dark}}} \sim 0$ (Figure 4.3C). This indicates that the presence of rise times is not related to the preceding adsorption event ($\tau_{\text{dark}}$) and is most likely associated with reorientation dynamics of the product molecule. More importantly, since rise times only occur for certain catalytic turnover events, it suggests that initial orientation of the product molecule during its formation could play an important role in the observed rotational dynamics.
Figure 4.3. (A,B) Autocorrelation functions of (A) $\tau_{\text{dark}}$ and (B) $\tau_{\text{rt}}$ between individual catalytic turnover events at 25°C on the same 5.4 nm Au nanoparticle. The dotted line in each autocorrelation plot indicates the 95% confidence intervals. (C) Scatter plots in log-log scale and cross correlations between $\tau_{\text{dark}}$ and $\tau_{\text{rt}}$ for individual 5 nm Au NPs at 25°C. All experiments are carried out at 4 µM resazurin and 1 mM NH₂OH.
4.3.3. Fluorescence quenching/enhancement effects as a function of preferred orientation of resorufin on Au surface

Our observation of rise times indicates an enhancement in fluorescence of resorufin which can be measured within our experimental time resolution of 45 msec. As a control experiment, we identified flowing resorufin solution over Au nanoparticles does not yield fluorescence bursts. This indicates that binding/unbinding of resorufin to Au nanoparticle, fluorescence blinking of Au nanoparticle-bound resorufin or free diffusion of resorufin, is not responsible for the observed fluorescent bursts and only the formation of a product molecule on the Au surface is responsible for the sudden increase in fluorescence intensity. The possible reasons that can contribute towards the observed slow fluorescence enhancement can be the relative position of the fluorescent molecule with respect to the Au surface, rotational dynamics of individual fluorescent molecules on the Au surface, and reversible electron transfer. Our single-molecule data indicates that the fluorescence bursts are concentrated at the same location (∼ 1 μm × 1 μm) over the experimental timeframe. If the distance between the resorufin molecule and the Au nanoparticle were to change, we would expect a change in fluorescence intensity between individual turnover events, or a complete loss of fluorescence as the molecule might not be on the Au surface (if the motion happens in z-direction). A simple calculation suggests the time taken for the resorufin molecule to travel to the evanescent wave region of 100 nm in the aqueous system (time, $t = L^2/D$, where $L$ is the distance traveled by the molecule and $D$ is the diffusion coefficient) is ∼ 23 μs (diffusion co-efficient of resorufin in water, $D = 4.34 \times 10^{-6}$ cm$^2$/s). Since our observation of both rise times and subsequent on-times is much larger than this timescale, we can assume that the resorufin molecule reorients on the Au surface upon its formation and diffuses away from the
excitation volume upon desorption. We hypothesize a dominant factor in this observed enhancement in fluorescence (slow rise in on-times) is related to the reorientation of the product molecule (rotational dynamics) on the Au surface upon its formation (Eq. 4). Once the product molecule achieves its preferred orientation, the fluorescence intensity reaches its maximum value and the period of $\tau_{on}$ starts. In cases where either the product molecule forms in its preferred orientation on the Au surface, or when it reorients to its preferred state within the time resolution of our experiments, we do not observe a rise time associated with $\tau_{off}$. The preferred orientation of the resazurin molecule on the Au surface is extremely difficult to determine using experimental methods. To better understand the preferred orientation of resorufin molecule on the Au surface and to determine the effects of fluorescence quenching/enhancement effect on rotational dynamics of resorufin with respect to the Au surface, we focus on theoretical calculations.

From DFT+D calculations, we determined the preferred orientation for both resazurin and resorufin on the Au (111) surface is with the aromatic ring parallel to the surface (Figure 4.4A). The optical dipole moment ($\mu_m$) of the $\pi$-conjugated part of the resorufin molecule is oriented perpendicular to the resorufin-nanoparticle axis and situated approximately 0.31 nm from the nanoparticle surface (Figure 4.4B). The orientation of the resazurin and resorufin is consistent with the reported binding modes for lissamine dye molecules\textsuperscript{10} and fluorescein derivatives\textsuperscript{19} on Au nanoparticles; both fluorescent molecules consist of a three aromatic ring structure similar to resorufin. In addition, we also changed the angle of resorufin from 0 to 90° with respect to the Au surface and determined the adsorption energy at each angle (Figure 4.5A-G). The distance between the resorufin-Au surface is maintained for the closest atoms while rotating the molecule. The energy at 90° corresponds to the highest-energy
adsorption state, with the plane of the molecule perpendicular to the Au surface. With the preferred orientation of resorufin determined from DFT+D calculations, we next determined the rate at which the product molecule emits fluorescence when it is present at a particular orientation with respect to the Au surface.
Figure 4.4. (A) Resorufin molecules interact with 5.4 nm Au nanoparticles through the nitrogen group. The distance between the optical dipole moment ($\mu_m$) of the $\pi$-conjugated part and the Au surface is 0.31 nm. (B) Results of DFT calculations indicate the preferred adsorption orientation of resorufin molecule on the Au (111) surface is with the molecular plane parallel to the Au surface. Nitrogen atoms are blue, oxygen atoms are red, hydrogen atoms are white, and carbon atoms are grey in color.
Figure 4.5. (A-F) DFT snapshots of orientation of the resorufin molecule at various angles (A) 10°, (B) 30°, (C) 50°, (D) 60°, (E) 70°, and (F) 90° on the Au (111) surface, when rotated about its nitrogen atom. (G) Normalized ΔG values at different orientations of resorufin on the Au (111) surface. All calculations were performed by maintaining the distance of 0.31 nm between resorufin and the Au surface.
The fluorescence yield of resorufin is affected by the orientation of the molecule with respect to the Au surface. A recent study by Anger et al.\textsuperscript{9} showed the continuous transition from fluorescence enhancement to quenching of a single fluorescent molecule as the molecule is brought closer to the Au surface. One possible reason for the quenching of fluorescence is the electron-transfer process from the excited molecule to the Au surface.\textsuperscript{9,10,19,20,41} However, the observation of fluorescence bursts during our single-molecule measurements indicate that the quantum yield of resorufin is sufficient enough to be detected experimentally. To probe further into the reorientation dynamics of resorufin, we determined the excitation rate, the quantum yield, and the fluorescence rate of resorufin as a function of molecular angle with respect to the Au nanoparticle surface through a collaboration with Prof. Lukas Novotny (ETH Zurich) (He performed all the calculations pertaining to the fluorescence rate based on the experimental data provided). This allows a direct comparison of fluorescence rate with the preferred orientation of the resorufin molecule from DFT calculations. The fluorescence rate ($\gamma_{em}$) of a single molecule on a metal surface can be expressed as a product of the excitation rate ($\gamma_{exc}$) and quantum yield ($q$).\textsuperscript{25} Quantum yield ($q$) is defined as the ratio of number of photons emitted to the number of photons absorbed. Treating the excitation and the emission process independently is legitimate because there is no coherence between the two processes. The fluorescence enhancement can then be expressed as:

\[
\frac{\gamma_{em}}{\gamma_{em}^o} = \frac{\gamma_{exc} q}{\gamma_{exc}^o q^o}
\]

where the superscript ‘o’ indicates the absence of the metal surface.
Let us consider a resorufin molecule interacting with a single spherical Au nanoparticle diameter (d = 5 nm). The resorufin molecule is oriented at an angle, $\theta$, with respect to the Au surface (Figure 4.6). The system is irradiated by a plane wave ($\lambda = 532$ nm) polarized along the z-axis. The excitation rate, the quantum yield as a function of molecule rotation on Au surface indicates maximum fluorescence when the plane of the molecule is oriented parallel to the Au surface (Figure 4.7A, B). The fluorescence rate of resorufin, which is a product of the excitation rate and quantum yield, also follows a similar trend (Figure 4.7C). The fluorescence rate of 0.0005 when the molecule is oriented parallel ($\theta = 0^\circ$) to the Au surface (at a distance of 0.3 nm) indicates that the fluorescence of resorufin is quenched 2000 times (more than its free state value) when bound to the surface. When the molecule detaches from the Au surface, i.e., the distance of the resorufin molecule is changed from 0.3 nm to infinity, the fluorescence rate goes to 1. The fluorescence rate is zero when the molecule is oriented perpendicular to the Au surface ($\theta = 90^\circ$), and a continuous transition to fluorescence enhancement occurs as the molecule is rotated from $90^\circ$ to $0^\circ$. The rotation dynamics of resorufin on the Au surface are most likely responsible for the observation of rise times and the resulting slow increase in fluorescence intensity observed in single-molecule measurements. A possible scenario for the observation of rise times is when the resorufin molecule forms on the Au nanoparticle surface at an angle and reorients itself to its most favorable configuration, thereby leading to an enhancement in its fluorescence.
Figure 4.6. Schematic representation of the model used for the calculation of the fluorescence rate of resorufin as a function of molecular angle. Resorufin is excited at $\lambda_{ex} = 532$ nm and emit at $\lambda_{em} = 581$ nm. The fluorescent molecule is placed at a distance of 0.3 nm from the surface of a spherical 5.4 nm Au NP in an aqueous medium.
Figure 4.7. Variation in (A) quantum yield, (B) excitation rate, and (C) fluorescence rate as a function of molecular angle between the fluorescent molecule and the Au nanoparticle. The excitation rate and fluorescence rate are normalized with their corresponding free-space values (i.e., in the absence of a Au nanoparticle). The trend in fluorescence rate indicates that the fluorescence is completely quenched when the molecule is perpendicular to the Au surface followed by an increase in fluorescence as the molecule approaches the most favorable adsorption configuration (i.e., parallel to the Au surface). The particle diameter, \( d \), is 5.4 nm, and the excitation wavelength is 532 nm.
4.3.4. Effect of temperature on the observed rise times at the single-molecule level

We have observed the presence of rise-times in all our temperature dependent single molecule data (5 - 45°C) (Figure 4.8A-F). The \(<\tau_{rt}\) were found to decrease with increasing temperature and, at higher temperatures (35 and 45°C), the \(<\tau_{rt}\) were less than the frame rate of the camera (45 msec); therefore, the product formation is characterized by an instantaneous increase in fluorescence intensity. Although the spontaneous rate at which a fluorescence molecule emits photons is primarily dependent on the oscillatory strength of the fluorescent molecule, studies have shown that the fluorescence rate is slightly influenced by environmental parameters, such as temperature and solvent polarity.\(^{23,42}\) The increase in temperature results in a decrease in the viscosity of water and could possibly accelerate the orientational dynamics of resorufin on the Au surface. This would lead to reduced rise times at higher temperatures consistent with our observation. In addition, the computed \(<\tau_{rt}\) exhibits Arrhenius behavior and results in an activation energy \((E_{a,rt})\) of 4.1 ± 0.4 kcal/mol (Figure 4.9). This calculated activation energy is similar in magnitude to the value of activation energy for the reaction \((E_{a,SM} = 4.6 ± 0.4\text{ kcal/mol})\) for the formation of resorufin, as determined in Chapter 2.
Figure 4.8. (A-F) Distributions of $\tau_{rt}$ from a single 5.4 nm Au nanoparticle trajectory at (A) 5°C, (B) 10°C, (C) 20°C, (D) 25°C, (E) 35°C, and (F) 45°C. Solid lines are exponential fits indicating the rise times follow a single-exponential decay. The average values of rise times are 0.133 s at 5°C, 0.107 s at 10°C, 0.096 s at 20°C, 0.07 s at 25°C, 0.03 s at 35°C, and 0.01 s at 45°C, respectively. All experiments are carried out in 4 μM resazurin and 1 mM NH$_2$OH.
Figure 4.9. Temperature dependence of $\ln \langle \tau_r \rangle^{-1}$ from single molecule experiments obtained by determining the $\langle \tau_r \rangle$ from fluorescence trajectories at each temperature. Solid line is a linear fit and the slope corresponds to activation energy for rise time ($E_{a,rt}$) of $4.1 \pm 0.4$ kcal/mol. Error bars are values determined from 3 different Au nanoparticles at 5°C, 5 different Au nanoparticles at 10°C, and 10 different Au nanoparticles at 20, 25, 35, and 45°C, respectively. All experiments are carried out in 4 μM resazurin and 1 mM NH$_2$OH.
It is particularly interesting to observe such a large activation barrier associated with rotation dynamics and that the values of activation energies between two different processes \((E_{a,SM} \text{ and } E_{a,rt})\) are of the same order of magnitude. However, it should be noted that the determination of \(E_{a,SM}\) assumes the rise times are incorporated in the observed \(\tau_{off}\) values. This could possibly contribute to the observation of similar activation energies for both processes. We will focus on the magnitude of the apparently high activation energy associated with product reorientation. A closer examination of the value of \(E_{a,rt}\) indicates that it represents the energy of a hydrogen bond.\(^{43}\) In a recent study by Akimov et al., they performed extensive UFF force field Molecular Dynamics (MD) simulations to investigate the rotational dynamics of single thioether molecules on Au (100) and Au (111) surfaces. Their result indicated that the dynamics of rotation of single thioether molecules of various alkyl chain length on Au (100) surfaces has an associated activation barrier of 2-3.5 kcal/mol.\(^{44}\) From the temperature dependence of the rotational diffusion coefficient, they determined the activation barriers of rotation. It was identified that the size, structure and chemical bonding of the anchor group were the most important factors influencing the observed rotation dynamics. For example, they considered an asymmetric thioether with one chain containing 6 carbon atoms and another chain containing only 4 carbon atoms. They identified that the first 4 groups on both chains will be compensated by interactions with the surface for each other, while the remaining two groups on the longer chain will have to spend some extra energy to overcome barriers in order for the whole molecule to rotate. For the Au (100) surface, it was determined that the molecule prefers to orient on top of the surface atoms. However, for the Au (111) surface, one CH\(_3\) group was located on the three-fold hollow site while another group was found on the atop site. The activation barrier is a result
of the difference between the saddle points and minima on a free-energy profile that the molecule explores during its rotation. They identified that the strongest repulsion between the CH$_3$ groups and the Au surface occurs when the group is on an atop site. Duration rotation, depending on the type of site, one CH$_3$ group passes through relative to the other results in the observed activation barriers for rotation. In our case, the product molecule, resorufin, consists of three benzene rings and a hydroxyl groups at each end. During its rotation, the molecule should overcome an activation barrier. This barrier for rotation of the resorufin molecule is most likely dependent on the relative position of the benzene rings with respect to the Au surface. A closer examination of the value of $E_{a,rt}$ indicates that it represents the energy of a hydrogen bond.

Through our single molecule measurements, we have identified the presence of rise times between individual catalytic turnover events and have correlated it with the reorientation dynamics of the resorufin on the Au surface. However, in the case of single molecule measurements, the experimental conditions need to be carefully manipulated in order to obtain single turnover resolution. Therefore, when one discusses single molecule data, it should be cross-examined by other experimental techniques, most preferably, the same system investigated at the ensemble level.

4.3.5. Determination of delay times from ensemble level measurements of resazurin reduction to resorufin

We have studied the ensemble-level kinetics of resazurin reduction to resorufin on Au nanoparticles using stopped-flow spectroscopy. The experiments were carried out at 1 msec time resolution and the data collected after the first 5 msec (to account for system’s dead
time) was analyzed. Figure 4.10 represents the observed fluorescence emission of resorufin at 5, 10, 20, 25, 35, and 45°C. All of the emission spectra collected indicates a characteristic delay that is present before the fluorescence of resorufin increases. We called the time for this characteristic delay the “delay time” ($\tau_{dt}$). The delay times are a function of temperature and decreases with increasing temperature. In addition, the experimental delay times were found to be similar to the observed rise-times at all temperatures.
Figure 4.10. (A) Ensemble-level stopped-flow measurements of fluorescence emission of resorufin at indicated temperatures. The plots show two distinct regions, a delay time followed by a linear increase in fluorescence. The solid lines are linear fits to the two distinct regions which are associated with the delay-times and the continuous fluorescence emission, respectively. The value of these delay times compare well with the rise times observed from single molecule measurements at the same temperature. It is observed that the delay times become progressively shorter at higher temperature. All ensemble-level measurements were carried out in 4 μM resazurin, 1 mM NH₂OH and 42 nM 5.4 nm Au nanoparticles.
In the case of the stopped-flow technique, the reactants are mixed rapidly in an observation chamber and the product formation is detected. This could lead to the notion that the presence of a delay before the fluorescence emission of resorufin is actually a direct result of the time taken for the reactants to mix together, resulting in the formation of the resorufin. However, if the product is instantaneously formed upon reactants mixing, it should be detected using the stopped-flow instrument. The validity of the previous statement can be verified by measuring the absorbance of resorufin for the same timeframe over which the fluorescence emission was measured. One of the advantages of the stopped-flow instrument we have utilized is that it allows simultaneous determination of absorbance and fluorescence emission of a fluorescent molecule. The absorbance spectra for resorufin indicate an instantaneous increase at all temperatures and no characteristic delay was observed (Figure 4.1). This confirms that the delay-time is present only during fluorescence emission after resorufin is formed on the Au surface and is consistent with our observation of rise-times in the case of single-molecule measurements. The delay time represents the average of all the initial dynamic processes that take place upon formation of resorufin on the Au surface. Since stopped-flow is an ensemble level technique, the value of the observed delay time is the average of all the values of rise times observed from an entire trajectory on multiple Au nanoparticles. The observed delay times also displayed Arrhenius behavior with an activation energy \( (E_{a,dt}) \) of 15.3 ± 2.7 kcal/mol (Figure 4.12A). This value is ~ 3 times higher than the value of \( E_{a,rt} \) observed from our single molecule measurements. Although \( E_{a,dt} \) appears to be similar to the value of ensemble-level activation energy \( (E_{a,app}) \) of 15.1 ± 0.8 kcal/mol observed in Chapter 2, we cannot directly compare the two values. In Chapter 2, based on the reaction rate expression, we deconvoluted this apparent activation energy \( (E_{a,app}) \) to be a sum
of two individual terms, the true activation energy ($E_{a,ens} = 4.6$ kcal/mol) and free energy of adsorption ($-\Delta G_{ads} = 9.3$ kcal/mol). Furthermore, we tested for the dependence of delay-times on resazurin concentration. Figure 4.12B indicates that the value of delay times is independent of resazurin concentration. This is similar to the observation during our single molecule measurements, wherein the value of $\tau_{rt}$ was found to be independent of resazurin concentration. Overall, the observation of delay times associated with fluorescence emission of resorufin is interesting and represents the initial reorientation dynamics of resorufin. In addition, these experiments also provide the necessary verification for the observation of rise times at the single-molecule level.
Figure 4.11. Ensemble-level stopped-flow measurements of absorption of resorufin at 570 nm at the indicated temperature. The timescales for each temperature is identical to the timescales used in Figure 4.10. Solid lines are linear fits illustrating the direct increase in absorbance of resorufin. The rate of increase in absorbance is slow at lower temperatures (5, 10°C) and the linear fit appears to have near zero slopes, but continued observation of absorbance indicate a steady increase and absence of a point of inflection when compared to the fluorescence emission plots. From the values of slope at 20, 25, 35, and 45°C, we determined the value of $E_{a,app} = 15.9$ kcal/mol. This value is similar to the value of $E_{a,app}$ reported from the ensemble level UV-Vis measurements.
Figure 4.12. (A) Temperature dependence of \( \ln <\tau_{dt}>^{-1} \) resulting in an activation energy \((E_{a,dt})\) of 15.3 ± 2.7 kcal/mol. Error bars are the S.D. resulting from multiple runs. All ensemble-level measurements were carried out in 4 µM resazurin, 1 mM NH\(_2\)OH and 43 nM 5.4 nm Au nanoparticles. (B) Stopped-flow measurements of fluorescence emission of resorufin at varying concentration of resazurin at 25 °C. The value of \( \tau_{dt} \) is independent of resazurin concentration. The concentrations 0.05, 0.1, and 0.2 µM indicate sub-saturation conditions while 1, 2, and 4 µM indicate saturating concentrations of resazurin.
4.4. Conclusions

In conclusion, we have demonstrated, through single molecule measurements and supporting theoretical calculations, one can probe the reorientation dynamics associated with a fluorescent dye molecule on a metal surface. The time resolution of the TIRF technique used for our single molecule measurements in this study is 45 msec. The presence of a slow rise in the fluorescence intensity of the product molecules upon its formation was observed during certain catalytic turnover events during the reduction of resazurin to resorufin on 5.4 nm Au NPs. By dissecting the individual contributions to the observed waiting times for product formation (τ_{off}) into τ_{dark} and τ_{rt}, we determined the major contributor to the observation of rise times (τ_{rt}) is the reorientation dynamics of the fluorescent product molecule (resorufin). The values of τ_{dark} and τ_{rt} between individual turnover events were found to be completely uncorrelated. Additionally, no correlation was found between a preceding τ_{dark} and the next immediate value, τ_{rt}. DFT calculations indicated that the most stable mode of adsorption for resorufin on Au (111) surface is with its aromatic ring parallel to the Au surface. To better quantify the change in fluorescence intensity of resorufin upon formation to its reorientation dynamics, we determined the fluorescence rate of resorufin as a function of the molecular angle of rotation with respect to the Au surface. It was observed that fluorescence intensity of resorufin increased as the angle was changed from 90° to 0° indicating that the molecule emits the maximum fluorescence when it is in its preferred mode of adsorption. From temperature-dependent single molecule measurements, an activation barrier for the reorientation dynamics was determined and most likely originates based on the relative position of the molecule with respect to the Au surface during the course of the reorientation process. Ensemble-level stopped-flow measurements indicated the presence of
delay times that were similar to the observed rise times at all temperatures. The delay times were observed only during fluorescence emission of resorufin and determined to be independent of the reactant concentration. The delay times represents an average of all the initial reorientation dynamics that are associated with resorufin molecules upon formation of resorufin on the Au surface and represents an ideal way to verify the observation of rise times at the single-molecule level.

4.5. References


Chapter 5

Thermodynamic Profiles at the Solvated Inorganic-Organic Interface: The case of Gold-Thiolate Monolayers

5.1. Introduction

The study of surface and interfacial properties at an organic-nanoparticle interface through the binding of suitable ligands is vital to obtaining desired functionalities for a wide range of applications in nanotechnology and nanobiotechnology.\textsuperscript{1-3} The presence of ligands can modulate the surface functionality of nanoparticles (NPs) and allows one to tailor interfacial and chemical properties such as stability, solubility, surface chemistry, binding affinity, and catalytic performance.\textsuperscript{4-10} The kinetic and thermodynamic contributions to the adsorption/self-assembly of organic molecules enables assessment of the relationships that exist between the head group interactions with the metal surface and the tail group interactions with adjacent adsorbates. The complexity associated with understanding these interactions arises because several of them occur simultaneously – hydrogen bonding, van der Waals interactions, solute-solvent interactions, and solvent reorganization which influence the structural details of the final organic-inorganic interface and the measured thermodynamics. These interactions exhibit thermodynamic behaviors that vary over a wide range of energy with both parallel and series kinetics that span several timescales. From an experimental standpoint, these interactions are not well-quantified in solvated systems since it is difficult to interrogate interfaces buried by solvent.\textsuperscript{11-16} Several studies have highlighted the difficulty in determining these contributions; the most pertinent ones by Jung et al.\textsuperscript{17}, Schessler et al.\textsuperscript{18}, and Karpovich et al.\textsuperscript{19} investigated the kinetics of alkanethiol adsorption
(and their kinetic results agreed in cases of overlap) while Bain et al.\textsuperscript{20} investigated kinetics of alkanethiol desorption and translated it to a thermodynamic description.

Self-assembled organic monolayers (SAMs) represent the quintessential nanoscale organic-inorganic interface because they provide an organic platform of controllable molecular thickness able to anchor different chemical species by a multitude of interactions.\textsuperscript{4,5,9,21-24} In addition, the tail group exposed to solvent can be modified to enhance the monolayer with new or additional functionality.\textsuperscript{25} Fundamentally, the self-assembly process relies on adsorption and molecular reorganization events. An understanding of the forces driving the self-assembly at an interface requires a comprehensive account of the thermodynamic parameters (\(\Delta H, \Delta S\)). We employ ITC to measure the equilibrium adsorption constant, enthalpy of adsorption, and adsorption stoichiometry in a single experiment. This allows for a full thermodynamic description of the thiol adsorption on the solvated gold nanoparticle (Au NP) interface, which is advantageous over an indirect approach, like van’t Hoff analysis, which requires multiple experiments conducted at various temperatures. In this paper, we report the influence of alkyl chain length, temperature, particle size, and solvent contributions on the thermodynamic description of carboxylic acid terminated thiol adsorption on Au NPs.

\textbf{5.2. ITC Theory}

ITC theory is well-developed in the literature for enzymatic studies, but we will briefly introduce the main concepts behind ITC theory and emphasize the mathematics that is most pertinent to our work. First, we consider the binding of a single type of ligand, L, with a metal receptor, M, in solution:
\[ M + nL \leftrightarrow ML_n \]  
(1)

where \( n \) represents the total number of ligands (i.e. the binding stoichiometry) that bind to the metal and \( ML_n \) is the final complex that forms between the metal and \( n \) ligands. We will only consider two types of binding within the scope of this work, though interested readers are referred elsewhere for additional information.\(^{26}\) The first type of binding is one-site independent binding, in which the metal may have several binding sites, but each site is thermodynamically identical and has the same thermodynamic affinity for the ligand. We can write the equilibrium binding constant, \( K_1 \), for the case \( n = 1 \) as

\[ K_1 = \frac{[ML]}{[M][L]} \]  
(2)

where the terms in brackets represent concentrations of the respective species. The second type of binding is multiple-sites binding, in which the metal has two thermodynamically distinct sites. Specifically, each site has its own affinity for the same ligand, but the occupancy of one site does not affect the affinity of the other, that is, the sites do not exhibit cooperative binding behavior. For our systems, we can model this behavior by considering the sequential binding of the same type of ligand to the same metal center twice \( n = 2 \):

\[ M + L \leftrightarrow ML \]  
(3)

\[ ML + L \leftrightarrow ML_2 \]  
(4)

For Eq. (3), the equilibrium constant is given by Eq. (2), but for Eq. (4), the equilibrium constant is given by

\[ K_2 = \frac{[ML_2]}{[ML][L]} \]  
(5)

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With full expressions for the respective equilibrium constants, it is now possible to combine these expressions with mass balances on each component:

$$[M]_T = [M] + [ML] + [ML_2]$$  \hspace{1cm} (6)

$$[L]_T = [L] + [ML] + 2[ML_2]$$  \hspace{1cm} (7)

Eqs. (6) and (7) can be extended to any binding system of \( n \) ligands, noting that \([M]_T\) and \([L]_T\) are the total concentrations of metal and ligand in the calorimeter cell. These mass balances can be substituted into the expressions for the equilibrium constants, such that only the total concentrations (i.e. measurable quantities) appear in the final ITC equations. This flexibility allows the experimenter to track the progress of the binding equilibria by calculating the molar ratio, the total amount of ligand in the calorimeter cell to the total amount of metal in the cell, as the independent variable. The dependent variable in ITC experiments is the total amount of heat released per injection of ligand, \(dQ\):

$$dQ = V \sum_i \Delta H_i d[ML_i]$$  \hspace{1cm} (8)

where \(V\) is the volume of the calorimeter cell, \(\Delta H_i\) is the enthalpy of binding for the formation of \(ML_i\), and \(d[ML_i]\) is the incremental amount of complex, \(ML_i\), formed during the injection. Eq. (8) may be extended to any number of complexes in solution. Substituting Eqs. (2) and (5)-(7) into Eq. (8) allow \(dQ\) to be written explicitly in terms of \(K_i, \Delta H_i, [M]_T,\) and \([L]_T,\) meaning that the heats from each injection can be fit to a statistical model as a function of the molar ratio that determines the binding parameters \((K_i, \Delta H_i, \text{and } n)\) in a single
experiment. The value of $\Delta G_i$ is determined from the standard thermodynamic definition, $\Delta G_i = -RT\ln(K_i)$, and the entropy of adsorption, $\Delta S_i$ is calculated from $\Delta G_i = \Delta H_i - T\Delta S_i$.

5.3. Experimental

5.3.1. Materials

Thioglycolic acid (C$_2$-TGA), 3-mercaptopropionic acid (C$_3$-MPA), and 6-mercaptohexanoic acid (C$_6$-MHA) were obtained from Sigma Aldrich (Milwaukee, WI). Gold nanoparticles ($5.4 \pm 0.7$, $9.5 \pm 0.6$, and $19.4 \pm 1.1$ nm) protected by citrate in aqueous dispersions were obtained from Ted Pella (Redding, CA). Milli-Q grade water ($18.2$ MΩ·cm) was used in all experiments. All chemicals were used as received for the ITC experiments.

5.3.2 ITC Experimental Procedure for Binding of Thiols to Au NPs

ITC experiments were performed using a NanoITC calorimeter (TA instruments) equipped with gold reference and sample cells ($V = 1.014$ ml). All titrations were carried out at $283.15$, $293.15$, $298.15$ or $303.15$ K using a $100$ μL syringe at a stirring rate of $250$ rpm. The sample cell contains an aqueous dispersion of different-sized Au nanoparticles and the reference cell contains Milli-Q water. All solutions were degassed by pulling a vacuum of 0.3-0.5 atm using a temperature-controlled degassing system (TA instruments) for a period of 10-15 min prior to titrations. Since our experimental system consists of thiol and gold nanoparticles, the cell was initially flushed with the thiol solution in order to bind all the available gold sites present in the cell with the thiol and thereby eliminate the interaction of thiol with the walls of the cell during an experiment. Titrations were run as an incremental series of injections of the appropriate thiol solution into the gold nanoparticle solution, and
the power compensation was measured as a function of time. The area under each peak is a measure of two effects: heat of mixing of the adsorbate and thiol binding to the surface of the nanoparticle. Experiments conducted under identical conditions with Milli-Q water (no Au NPs) in the sample cell yielded experimental values for the heat of mixing. The heat of mixing data was subtracted from the experiments with Au NPs in the cell and integrated to isolate the heat evolved from thiol-NP interactions. Data analysis was performed using the NanoAnalyze software from TA instruments using an independent model (see ITC theory section for derivation of appropriate model)\textsuperscript{27}. We used a modified version of the multiple-sites model in Microsoft Excel in order to account for the inability of NanoAnalyze to fit integrated heat data near zero accurately. The Solver function in Excel was employed to minimize the sum of the squares of the differences between the measured heat and the calculated heats. Error analysis was performed using NanoAnalyze via its Statistics function. The uncertainties in the parameters obtained from the fits are calculated by adding perturbations to the optimized fits and then refitting the model for a set number of trials. Each perturbation obeys a Gaussian distribution that has the same standard deviation generated from the original fit. The error in each data set was determined within one standard deviation for 1000 trials. The error values reported in the main text for the $K$ values are also multiplied by the factor outside of the parentheses. For example, a $K$ value of $(3.3 \pm 0.5) \times 10^6$ means that the error is $0.5 \times 10^6$.

5.3.3. Solution Calorimetry

Solution calorimetry experiments were performed on a TAM III microcalorimeter (TA Instruments) at 298.15 K. Samples of pure C$_2$-TGA, C$_3$-MPA, and C$_6$-MHA were placed into glass ampoules and sealed with wax to prevent premature mixing of water with
the thiols. Sealed ampoules were immersed in 25 mL of milli-Q water in a reaction cell and stirred at 600 rpm. Ampoules were then broken and the thiols was allowed to mix with the solvent for 1 h while monitoring the heat flow. An electronic heat pulse was applied to the reaction cell before and after dissolution to calibrate the heat capacity in each instance. An empty ampoule (blank) was broken to account for the heat evolved due to breaking the glass (This value is typically minimal). The total heat evolved or absorbed during each experiment was obtained using the Analyze Experiment function in the SolCal v1.2 software (TA Instruments). Each heat value was then corrected for the blank experiments and normalized to the total amount of moles of solute mixed to calculate the enthalpy of solvation.

5.3.4. Thermogravimetric Analysis

Thermal gravimetric analysis (TGA) was performed on a Netzsch TG 209 F1-Iris Thermogravimetric analyzer under a nitrogen atmosphere (20 ml/min). Gold nanoparticles saturated with C₃-MPA were centrifuged, separated and dried in an oven (at 80 °C for 16 h). Dried Au samples (1-2 mg) were deposited onto Al crucibles as powders and placed in the instrument until a stable weight was obtained prior to analysis. The samples were heated at a rate of 10 °C/min up to 100°C, held at that temperature for 10 min to ensure evaporation of all the solvent, and then heated to 600°C at the same rate.

5.3.5. Determination of Au surface atom (Auₖ) concentration

The thiol atoms bind only to gold surface atoms and the core atoms do not participate in the adsorption process. In order to represent the true concentration of available sites for binding in an ITC experiment, it is essential to determine the surface gold concentration rather than the bulk concentration. The nanoparticle density (# of nanoparticles/ml) is known
from Ted Pella Inc. for the 5, 10, and 20 nm gold nanoparticles utilized in our experiments. For spherical particles, the radius of the nanoparticle can be expressed as

\[ R_{NP} = R_{atom} \times N_t^{1/3} \]  

(9)

where \( R_{NP} \) is the radius of the respective nanoparticle, \( R_{atom} \) is the atomic radius of gold (1.74 Å), and \( N_t \) is the total number of gold atoms in a nanoparticle. The number of gold surface atoms (\( N_s \)) can then be obtained from

\[ N_s = \frac{\text{Surface area of nanoparticle}}{\text{Cross Sectional Area of individual atom}} = \frac{4\pi(R_{NP})^2}{\pi(R_{atom})^2} = 4 \times N_t^{2/3} \]  

(10)

By calculating the total number of moles of surface atoms present in 1.014 ml (ITC cell volume) of gold nanoparticle solution, we can estimate the concentration of gold surface atoms present in the cell. The concentration of gold surface atoms for 5.4, 9.5, and 19.4 nm gold nanoparticles was determined to 0.08, 0.04, and 0.02 mM, respectively. The total concentration of gold atoms for 5.4, 9.5, and 19.4 nm correspond to 0.32, 0.29, and 0.28 mM respectively.

In addition, according to the statistics of surface atoms for a cuboctahedral particle developed from van Hardeveld and Hartog\textsuperscript{28}, we estimated the number of Au surface atoms located on (111) and (100) facets for each NP size and is represented in Table 5.1.
Table 5.1. Statistics of Au surface atoms on 5, 10, and 20 nm Au NPs

<table>
<thead>
<tr>
<th>Au NP diameter (nm)</th>
<th>Total number of Au atoms</th>
<th>Au (111) surface atoms</th>
<th>Au (100) surface atoms</th>
<th>Au corner and edge atoms</th>
<th>% Au (111) surface atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3860</td>
<td>848</td>
<td>210</td>
<td>120</td>
<td>71.9</td>
</tr>
<tr>
<td>10</td>
<td>30896</td>
<td>3440</td>
<td>858</td>
<td>264</td>
<td>75.4</td>
</tr>
<tr>
<td>20</td>
<td>247168</td>
<td>12680</td>
<td>3168</td>
<td>528</td>
<td>77.4</td>
</tr>
</tbody>
</table>

5.3.6. Determination of Coverage (θ)

In ITC, for a given initial concentration of the metal and thiol in the cell and syringe, respectively, the total concentration of metal and thiol in the calorimetric cell after the $k^{th}$ injection is given by

$$[M]_k = [M]_0 \left(1 - \frac{V_k}{V_0}\right)^k$$

(11)

$$[L]_k = [L]_0 \left(1 - \left(1 - \frac{V_k}{V_0}\right)^k\right)$$

(12)

where $[M]_0$ and $[L]_0$ are the initial metal concentration in the cell and the concentration of thiol in the syringe, respectively, and $V_k$ and $V_0$ are the injection volume and cell volume, respectively. The concentration of the metal-thiol complex at any point in the calorimetric cell is given by,

$$[ML] = \frac{1}{2} \left(\frac{[M]_k}{n} + \frac{[L]_k}{n} + \frac{1}{nK_{ads}}\right) - \sqrt{\left([M]_k + \frac{[L]_k}{n} + \frac{1}{nK_{ads}}\right)^2 - 4[M]_k[L]_k}$$

(13)
where \( n \) is the stoichiometric binding ratio and \( K_{ads} \) is the adsorption constant obtained from ITC experiments. The coverage (\( \theta \)) after each injection \( k \) can be determined from

\[
\theta = \frac{[\text{ML}]_k}{[\text{M}]_k}
\]

(14)

The heat absorbed or evolved during each injection (\( \Delta Q \)) is proportional to the change in concentration of the formed complex (\( \Delta [\text{ML}] \)), the molar enthalpy of binding (\( \Delta H_{ads} \)), and the volume of the calorimetric cell (\( V_0 \)):

\[
\Delta Q = n\Delta [\text{ML}] \Delta H_{ads} V_0
\]

(15)

Eqs. (11-15) are sufficient to determine the variation of \( \Delta H_{ads} \) versus coverage (\( \theta \)).

5.4. Results and Discussion

5.4.1. ITC results for binding of C\(_2\)-TGA, C\(_3\)-MPA, and C\(_6\)-MHA to Au NPs

Figures 5.1 and 5.2 are thermograms for binding of thioglycolic acid (C\(_2\)-TGA), 3-mercaptopropionic acid (C\(_3\)-MPA), and 6-mercaptohexanoic acid (C\(_6\)-MHA) to 5.4 ± 0.7 nm Au NPs at 298.15 K along with the integrated heats and the corresponding best-fit binding models for each system as measured by ITC. Titrations were run as an incremental series of injections of the appropriate thiol solution into the Au NP solution, and the power compensation was measured as a function of time. The area under each peak is a measure of two effects: heat of mixing of the adsorbate and thiol binding to the surface of the NP. The ITC experimental conditions are summarized in Table 5.2, the best-fit parameters obtained from an independent model are presented in Table 5.3, and the parameters obtained from a multiple-sites model are presented in Table 5.4. In the case of independent model, the metal
may have several binding sites, but each site is thermodynamically identical and has the same thermodynamic affinity for the ligand. For multiple-sites model, the metal has thermodynamically different types of sites. Specifically, each site has its own affinity for the same ligand, but the occupancy of one site does not affect the affinity of the other, that is, the sites do not exhibit cooperative binding behavior.\textsuperscript{26} The experiments were carried out at a pH of 6.1 (intrinsic pH of Au NP solution), and the binding of \textit{C}_{2}-\textit{TGA} to Au NPs was repeated at pH 4 and 7. The two pH values of 4 and 7 were chosen to provide different concentration of dissociated carboxylic acid species on the Au surface. Solutions of 1 N HCl and 0.1 N NaOH were used to adjust the pH of the thiol and Au NP solutions to the desired values. We constructed the speciation diagram for \textit{C}_{2}-\textit{TGA} by utilizing the pK\textsubscript{a} from aqueous solution data, which indicates that at a pH of 6.5, the concentration of COOH and COO\textsuperscript{-} are similar (Figure 5.3). To construct a speciation diagram for carboxylic acid group dissociation on \textit{C}_{2}-\textit{TGA}, it is important to know the different complexes that are formed with their corresponding reaction governed by its equilibrium constant.

\[
\text{RCOO} + H^+ \overset{K_1}{\leftrightarrow} \text{RCOOH}
\]  

The value of log \( K_1 \) (for Eq. 16) for \textit{C}_{2}-\textit{TGA} dissociation is 3.44 \( \pm \) 0.04.\textsuperscript{29}

The stoichiometry (\( n \)) value suggests that \textit{C}_{2}-\textit{TGA} and \textit{C}_{3}-\textit{MPA} prefer binding to three Au surface atoms (Au\textsubscript{s}), a stoichiometry consistent with the three-fold site binding of thiols of Au NPs.\textsuperscript{30} We carried out thermogravimetric experiments using \textit{C}_{3}-\textit{MPA} on 5.4 \( \pm \) 0.7 and 9.5 \( \pm \) 0.6 nm Au NPs and determined the stoichiometry of binding of \textit{C}_{3}-\textit{MPA} to the Au NPs. Figures 5.4A and 5.4B represent the reduction in weight of Au NPs saturated with \textit{C}_{3}-\textit{MPA} as a function of temperature. From the analysis of the TGA data, the ratio of \textit{C}_{3}-\textit{MPA} molecules to Au surface atoms was determined to be 0.29 for 5.4 \( \pm \) 0.7 nm and 0.27 for
9.5 ± 0.6 nm Au nanoparticles, respectively. This agrees well with the value of stoichiometric binding ratio obtained from ITC experiments. Our thermogravimetric analysis confirms the three-fold hollow site binding mode for C₃-MPA. Our results indicate a higher stoichiometry (n = 0.2) in the case of C₆-MHA (Table 5.3). We believe that this is a result of certain Au sites in the vicinity of the primary adsorption hollow site that are sterically hindered by the presence of adsorbed C₆-MHA, while this appears not to be the case for the shorter-chain thiols, C₂-TGA and C₃-MPA.
Figure 5.1. Real-time ITC thermograms for C$_2$-TGA at pH (A) 4, (B) 6.1, and (C) 7 binding to 5.4 ± 0.7 nm Au NPs at 298.15 K with (D), (E), and (F) as the respective integrated heat data with fitted models. (D) is fit using multiple-sites model, (E) is fit using both multiple-sites and independent models, while (F) is fit using an independent model. “Q” refers to the thermal compensation of the calorimeter to keep the sample at a constant temperature (positive peaks are exothermic). Each peak corresponds to a single injection of C$_2$-TGA solution into the nanoparticle suspension.
Figure 5.2. Real-time ITC thermograms at pH 6.1 for (A) C₃-MPA and (B) C₆-MHA binding to 5.4 ± 0.7 nm Au NPs at 298.15 K with (C) and (D) as the respective integrated heat data with an independent model fit.
Table 5.2. Experimental conditions for ITC experiments on different nanoparticle sizes. For each experiment listed, the gold nanoparticle and thiol concentrations are provided, along with the volume of each injection ($V_{\text{inj}}$), the total number of injections ($N_{\text{inj}}$), and the duration of each injection ($t_{\text{inj}}$) which refers to the amount of time that elapses after an injection in order to allow the baseline to equilibrate.

<table>
<thead>
<tr>
<th>Thiol</th>
<th>NP size</th>
<th>[Au] (mM)</th>
<th>[Ligand]</th>
<th>$V_{\text{inj}}$</th>
<th>$N_{\text{inj}}$</th>
<th>$t_{\text{inj}}$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_2$-TGA</td>
<td>5.4</td>
<td>0.08</td>
<td>0.5</td>
<td>6</td>
<td>16</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>0.04</td>
<td>0.2</td>
<td>6</td>
<td>16</td>
<td>800</td>
</tr>
<tr>
<td>C$_3$-MPA</td>
<td>5.4</td>
<td>0.08</td>
<td>0.5</td>
<td>6</td>
<td>16</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>0.04</td>
<td>0.3</td>
<td>6</td>
<td>16</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>19.4</td>
<td>0.02</td>
<td>0.15</td>
<td>5</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>C$_6$-MHA</td>
<td>5.4</td>
<td>0.08</td>
<td>0.4</td>
<td>6</td>
<td>16</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>0.04</td>
<td>0.2</td>
<td>5</td>
<td>19</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>19.4</td>
<td>0.02</td>
<td>0.1</td>
<td>6</td>
<td>16</td>
<td>800</td>
</tr>
</tbody>
</table>
**Table 5.3.** Temperature dependence of the adsorption constant ($K$), Gibbs free energy of adsorption ($\Delta G$), enthalpy of adsorption ($\Delta H$), entropy of adsorption ($T\Delta S$), and molar heat capacity ($\Delta C_p$) of C$_2$-TGA, C$_3$-MPA, and C$_6$-MHA on 5.4 ± 0.7 nm Au nanoparticles at a pH of 6.1

<table>
<thead>
<tr>
<th>Thiol</th>
<th>$T$ (K)</th>
<th>$K$ (M$^{-1}$)</th>
<th>$\Delta G$ (kcal/mol)$^a$</th>
<th>$\Delta H$ (kcal/mol)$^b$</th>
<th>$T\Delta S$ (kcal/mol)$^a$</th>
<th>$n$</th>
<th>$\Delta C_p$ (kcal/mol.K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_2$-TGA</td>
<td>283.15</td>
<td>(1.4 ± 1.2) $\times$ 10$^7$</td>
<td>-9.3 ± 0.8</td>
<td>-23.6 ± 2.2</td>
<td>-14.4 ± 1.4</td>
<td>0.26 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>293.15</td>
<td>(1.3 ± 0.9) $\times$ 10$^7$</td>
<td>-9.5 ± 0.9</td>
<td>-20.5 ± 1.2</td>
<td>-10.9 ± 0.9</td>
<td>0.33 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>298.15</td>
<td>(5.1 ± 1.3) $\times$ 10$^6$</td>
<td>-9.1 ± 1.1</td>
<td>-20.0 ± 1.3</td>
<td>-10.9 ± 1.2</td>
<td>0.33 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>303.15</td>
<td>(4.2 ± 1.1) $\times$ 10$^6$</td>
<td>-9.2 ± 0.9</td>
<td>-18.4 ± 1.1</td>
<td>-9.2 ± 1.1</td>
<td>0.34 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>C$_3$-MPA</td>
<td>283.15</td>
<td>(8.5 ± 0.7) $\times$ 10$^6$</td>
<td>-8.9 ± 0.7</td>
<td>-22.8 ± 0.2</td>
<td>-13.9 ± 0.7</td>
<td>0.27 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>293.15</td>
<td>(5.7 ± 0.6) $\times$ 10$^6$</td>
<td>-9.0 ± 0.2</td>
<td>-21.2 ± 0.7</td>
<td>-12.2 ± 0.2</td>
<td>0.30 ± 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>298.15</td>
<td>(3.3 ± 0.5) $\times$ 10$^6$</td>
<td>-8.9 ± 0.4</td>
<td>-20.3 ± 1.1</td>
<td>-11.4 ± 0.4</td>
<td>0.37 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>303.15</td>
<td>(3.1 ± 0.4) $\times$ 10$^6$</td>
<td>-9.0 ± 0.5</td>
<td>-13.7 ± 0.6</td>
<td>-4.7 ± 0.5</td>
<td>0.30 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>C$_6$-MHA</td>
<td>283.15</td>
<td>(8.9 ± 0.1) $\times$ 10$^6$</td>
<td>-9.0 ± 0.1</td>
<td>-34.4 ± 1.2</td>
<td>-25.4 ± 0.1</td>
<td>0.20 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>293.15</td>
<td>(5.9 ± 0.7) $\times$ 10$^6$</td>
<td>-9.1 ± 0.6</td>
<td>-30.6 ± 0.5</td>
<td>-21.5 ± 0.6</td>
<td>0.16 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>298.15</td>
<td>(2.7 ± 0.4) $\times$ 10$^6$</td>
<td>-8.8 ± 0.2</td>
<td>-28.6 ± 0.2</td>
<td>-19.8 ± 0.2</td>
<td>0.20 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>303.15</td>
<td>(1.2 ± 0.6) $\times$ 10$^6$</td>
<td>-8.4 ± 0.2</td>
<td>-26.8 ± 0.7</td>
<td>-18.4 ± 0.2</td>
<td>0.24 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The values for $\Delta G$ and $T\Delta S$ were calculated from the $K$ and $\Delta H$ values.

$^b$The integrated heats for each titration were analyzed using the independent model to obtain binding constants, enthalpies of binding, and binding stoichiometries. Errors were calculated using the statistics function in NanoAnalyze.
Table 5.4. pH dependence of the association constant \( (K) \), Gibbs free energy of adsorption \( (\Delta G) \), enthalpy of adsorption \( (\Delta H) \), and entropy of adsorption \( (T\Delta S) \) of \( C_2\text{-TGA} \) on \( 5.4 \pm 0.7 \) nm Au nanoparticles (0.08 mM Au\(_s\)) at 298.15 K.

<table>
<thead>
<tr>
<th>pH</th>
<th>( K_1 ) (M(^{-1}))</th>
<th>( K_2 ) (M(^{-1}))</th>
<th>( \Delta H_1 ) (kcal/mol)(^b)</th>
<th>( \Delta H_2 ) (kcal/mol)(^b)</th>
<th>( T\Delta S_1 ) (kcal/mol)(^a)</th>
<th>( n_1 )</th>
<th>( n_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>(6.2 ± 0.7) × 10(^5)</td>
<td>-11.4 ± 0.7</td>
<td>-3.5 ± 0.4</td>
<td>0.28 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>(4.8 ± 0.5) × 10(^8)</td>
<td>-6.5 ± 0.5</td>
<td>5.3 ± 0.5</td>
<td>0.19 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>(6.4 ± 0.6) × 10(^6)</td>
<td>-29.8 ± 0.8</td>
<td>-20.5 ± 0.6</td>
<td>0.29 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>(1.1 ± 0.4) × 10(^6)</td>
<td>-10.3 ± 0.5</td>
<td>-2.1 ± 0.5</td>
<td>0.27 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>(7.2 ± 0.6) × 10(^6)</td>
<td>-11.2 ± 0.6</td>
<td>-1.9 ± 0.5</td>
<td>0.4 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)The values for \( \Delta G \) and \( T\Delta S \) were calculated from the \( K \) and \( \Delta H \) values.

\(^b\)The integrated heats at pH 4 and 6.1 for each titration were analyzed using the multiple-sites model to obtain binding constants, enthalpies of binding, and binding stoichiometry, while at pH 7, the independent model was used to obtain \( K \) and \( \Delta H \). Errors were calculated using the statistics function in NanoAnalyze.
Figure 5.3. Speciation diagram for C$_2$-TGA in solution shows the percent of each species as a function of solution pH.
Figure 5.4. Thermogravimetric analysis data for (A) 5.4 ± 0.7 nm and (B) 9.5 ± 0.6 nm Au NPs saturated with C₃-MPA.
The ITC thermogram represented in Figure 5.1A can be separated into three regions which reveal the distinct stages of thiol monolayer formation at the organic-inorganic interface. The first section, prior to the point of inflection, represents the adsorption of thiol on the Au NP surface and the formation of Au-S bonds. The non-linear region of the sigmoid indicates the occupation of the last available gold sites and simultaneous ordering of the thiol molecules on the surface since the coverage is approaching saturation. The tail section of the sigmoid refers to the heat of mixing of the thiols with water since no/minimal adsorption of thiol occurs at this point. The ITC thermograms in Figures 5.1 and 5.2 indicate that while all three thiols studied demonstrate independent site binding, there are changes in alkyl chain reorganization during the initial adsorption of C$_2$-TGA that leads to the observed changes in the thermograms (Figure 5.1A-C). At low coverages, the highly-disordered C$_2$-TGA monolayer reorganizes in response to the increasing chemical potential of C$_2$-TGA in the liquid-phase due to additional injections. The rate associated with this restructuring is highly dependent on the conformation of C$_2$-TGA molecules on the Au surface; we ensured each data reached equilibrium because subsequent injections were introduced to the calorimeter only after the baseline had equilibrated. C$_2$-TGA restructuring appears to be dependent on the solution pH. The binding of C$_2$-TGA to the 5.4 ± 0.7 nm Au NPs at a pH of 4.0 and 6.1 show a clear increase in the heats prior to the non-linear section of the sigmoid (Figure 5.1D and E), while no such behavior is observed at a pH of 7 (Figure 5.1F). The data at pH 4 and 7 was fit using a multiple-sites and an independent model, respectively, while the data at pH 6.1 was fit using both. C$_3$-MPA and C$_6$-MHA binding isotherms were fit using an independent model. The multiple-sites model provides insight into the electrostatic interaction (occurring at the front end of the sigmoid) and the ordering of C$_2$-TGA molecules on the Au NP surface,
while the thermodynamic parameters of adsorption were obtained from a one-site independent model and presented in Table 5.3. In the case of multiple-sites model, we do not know the actual meaning of the thermodynamic parameters \((K_1, \Delta H_1)\) obtained from the fit (Table 5.4), because we do not know the contribution of adsorption and reorganization events to the observed heat separately. One of the shortcomings of ITC is the inability to assign the heat flow to specific chemical events. Complemented by supporting analytical techniques that probe the chemistry occurring or by literature observations, the origin of heat flow can be correlated with chemical events. We have utilized observations from the literature to suggest the behavior in the initial position of the isotherm is due to C\(_2\)-TGA adsorption-restructuring events.\(^{31}\) It would be very difficult to deconvolute the effect of binding and monolayer restructuring from the ITC data.

5.4.2. Effect of pH on binding of C\(_2\)-TGA to Au NP surface

The observed trend in the ITC thermogram for C\(_2\)-TGA adsorption at different pH can possibly be explained by the electrostatic interactions at the front end of the sigmoid and the degree of dissociation of COOH groups. The acidity of C\(_2\)-TGA strongly decreases after adsorption (the p\(K_a\) of C\(_2\)-TGA in aqueous solution is 3.4;\(^{29}\)) and, in aqueous media, the COOH groups will begin to deprotonate to carboxylates (at a pH value of 5). The average conformation of C\(_2\)-TGA molecules adsorbed on the Au surface strongly depends on the degree of dissociation. Krolikowska et al.\(^{31}\) identified through surface enhanced Raman scattering (SERS) experiments that an increase in the number of COO\(^-\) groups on the Au surface leads to a highly disordered SAM. In addition to the effect of the COOH groups, the chloride (introduced due to pH adjustment) and citrate (used for the original synthesis of Au NPs) ions also interact with the Au surface. At pH of 4, there is an increased concentration of
chloride ions in solution, in addition to the citrate ions (they impart a net negative charge) present on the surface of the Au NPs. It is well-known that chlorine prefers to bind in either a three-fold hollow or a bridge site on the Au surface.\textsuperscript{32} While these anions may impact adsorption, it is also well-known that short chain-length thiols form disordered monolayers at low coverage, and ultimately reorganize with increasing thiol coverage. We believe the ITC thermogram captures these interactions in the front end of the sigmoid. This is most likely related to the reorganization of the monolayer due to favorable intermolecular interactions between adsorbates and repulsion of like-charge species on the Au surface. At low thiol coverages, C\textsubscript{2}-TGA adsorbs nearly parallel to the surface.\textsuperscript{31} As $\theta_{\text{thiol}}$ increases with successive injections, C\textsubscript{2}-TGA will adsorb tilted because crowding at the Au surface minimizes like-charge repulsion and leads to the presence of a sigmoid in an ITC thermogram. Similar observations have been made on Au (111) surface by Raman vibrational spectroscopy where C\textsubscript{2}-TGA was found to lie down on the substrate at low coverages and tilt towards the surface normal at high coverages respectively.\textsuperscript{33} Consistent with previous STM measurements\textsuperscript{34}, Jung et al.\textsuperscript{17} reported that in cases of alkanethiol adsorption, as adsorption proceeds, the thiols were observed to reside in two phases on the Au surface: a low-density lying down phase consisting of physisorbed alkanethiol species at low coverages, and a densely-packed upright phase of adsorbed alkanethiols at high coverages. This two-phase model can be extended to short-chain alkanethiol adsorption and our observations from ITC thermograms for C\textsubscript{2}-TGA adsorption on Au NPs appear to support this model. Due to C\textsubscript{2}-TGA speciation as well as decreased concentration of chloride ions at pH of 6.1, the density of negative charge is decreased on the surface, but the resultant thermogram still indicates like-charge repulsion and monolayer reorganization at the front end of the sigmoid. Although we cannot
identify what the obtained thermodynamic parameters ($K_1$, $\Delta H_1$) from the multiple-sites model fit represents, we demonstrate that ITC can measure complex interactions occurring at the front end of the sigmoid. At pH of 7, the density of negative charge is further reduced, and the data fits a one-site independent model (Figure 5.1F).

As the alkyl chain length increases, the acidity of the carboxylic groups decreases which is indicated by an increase in the pK$_a$ values. It results in a decreased concentration of deprotonated groups on the Au surface and an increased attractive interaction between the alkyl chains resulting from van der Waals interactions. This counteracts the repulsive interaction between the deprotonated carboxylic acid groups, resulting in an adsorbed thiol-NP surface with a greater degree of structure at low coverages. Thus, the near perfect sigmoid seen for the binding of C$_3$-MPA and C$_6$-MHA to Au NPs (Figure 5.2C and 5.2D) is most likely due to the direct adsorption of the thiol with minimal reorganization.

5.4.3. Influence of temperature on the thermodynamic parameters of binding of C$_2$-TGA, C$_3$-MPA, and C$_6$-MHA to Au NPs

Ligand exchange (i.e., reaction between free ligand and nanoparticle surface) is an important process to prepare functionalized nanoparticles. Understanding the thermodynamics of adsorption will also provide critical insight into the role of thiols during NP synthesis since thiols are a common ligand choice (i.e., Brust method). We believe that calorimetric studies of thiol exchange at higher temperatures should improve our knowledge on how these small molecules influence NP growth during colloidal synthesis. The thermodynamic information obtained from ITC experiments can be used to quantify the effects the various thiols have on the observed energetics at the thiol-NP interface. The
adsorption constants of C$_2$-TGA, C$_3$-MPA, and C$_6$-MHA on 5.4 ± 0.7 nm Au NPs indicate a strong affinity for binding (large values of $K$ from Table 5.3). The value for $\Delta G$ is similar for the three thiols at all temperatures analyzed (Table 5.3). Jung et al.\textsuperscript{17} determined that each added –CH$_2$– group stabilizes the adsorbed thiol by 0.35 kcal/mol in free energy for alkanethiol adsorption on Au from ethanol solution at 298.15 K and our results for C$_2$-TGA and C$_3$-MPA adsorption on 5.4 ± 0.7 nm Au NPs (added –CH$_2$– group stabilizes by 0.23 kcal/mol) are consistent with their results. The observed overall $\Delta G$ is similar at different conditions, therefore, further dissection of $\Delta G$ into enthalpic and entropic contributions is paramount for a complete understanding of the thiol-NP binding energetics. The $\Delta H$ and $\Delta S$ values provide insight as to what the dominant effects are for a given set of thiol-NP interactions. The subtlety of ITC measurements is that the obtained parameters are observed or apparent values, rather than intrinsic binding values. The observed $\Delta H$ includes the adsorption of the thiols, the loss/gain of van der Waals interactions (~ 1-2 kcal/mol per –CH$_2$– group)$^{17,36}$, hydrophilic/hydrophobic interactions at the organic-inorganic interface, hydrogen bonding between adjacent thiol molecules, loss of solute-solvent bonds in the form of solvent reorganization, and competitive adsorption between thiol and solvent.$^{17,30}$ The observed entropy at a particular temperature associated with thiol-NP binding can include contributions from solvation and desolvation of the thiol molecules, expulsion of water molecules ordered near the NP surface, and ordering of water molecules around hydrophobic alkyl chains (hydrophobic effect); however, the main contribution to the observed entropy is the loss of at least one degree of translational freedom of thiol molecules, hindered alkyl rotation and reduced trans-gauche interconversion upon adsorption, which normally manifests itself as a decrease in entropy.$^{37,38}$
The adsorption of C₂-TGA, C₃-MPA, and C₆-MHA on 5.4 ± 0.7 nm Au NPs became progressively stronger with decreasing temperature; this is indicated by an increase in the magnitude of $\Delta H$ values. From Table 5.3, the binding of all thiols is enthalpy-driven at all temperatures because of the large exothermic enthalpies. In addition, the coverage ($\theta$) and differential enthalpy of adsorption ($\Delta H_{\text{diff}}$) were determined as a function of injection number for the three thiols used and represented in Figure 5.5. For all three thiols, with increasing $\theta$, there is a rapid decrease in $\Delta H_{\text{diff}}$ until the saturation point. A subsequent increase in $\theta$ leads to a very small change in the value of $\Delta H_{\text{diff}}$. During the first injection, thiol molecules bind strongly to the most active Au sites. With successive injections, the thiol molecules bind to the available sites with decreasing strength (i.e., a less negative $\Delta H_{\text{diff}}$). The enthalpies of solvation ($\Delta H_{\text{solv}}$) for pure C₂-TGA, C₃-MPA, and C₆-MHA were determined using solution calorimetry to be 0.86, 1.17, and 0.55 kcal/mol, respectively. The $\Delta H_{\text{solv}}$ is endothermic for all thiols, but demonstrates a unique trend as a function of carbon number that is unexplained at this time. For the three cases, the observed $\Delta H_{\text{solv}}$ is negligible as the thiols are solvated prior to the experiments, and does not contribute significantly to the observed $\Delta H$ values. From a thermodynamic standpoint, the self-assembly of thiols on Au NPs is driven by the enthalpy factor $\Delta H$ due to the formation of a highly stable Au-S bond.
Figure 5.5. Differential enthalpy of adsorption ($\Delta H$) with coverage for (●) C$_2$-TGA, (■) C$_3$-MPA, and (♦) C$_6$-MHA on 5.4 ± 0.7 nm Au nanoparticles at 298.15 K.
At any given temperature, the observed $\Delta S$ is negative and with decreasing temperature, we observe that $\Delta S$ contributes more unfavorably to $\Delta G$, i.e., $\Delta S$ values become more negative. It is extremely difficult to measure the intrinsic entropy contribution to the free energy of thiol-Au association directly in solution. However, it is important to understand that due to the loss of translational and rotational degrees of freedom upon covalent bond formation, the entropy change should be significantly negative.\textsuperscript{39} Searle and Williams estimated an entropy cost of -7 to -10 kcal/mol (in $T\Delta S$ units at 298.15 K) for binding of small molecules to proteins in solution due to the loss of rigid-body entropy, noting that larger losses in entropy are associated with larger increases in enthalpy, i.e., enthalpy-entropy compensation.\textsuperscript{40} Upon adsorption, a molecule cannot lose more entropy than it possesses (i.e., its absolute entropy) and therefore, the intrinsic $\Delta S$ due to adsorption must be negative and must have a magnitude smaller than its absolute entropy in the gas phase $S_g^o$.\textsuperscript{41} Our results indicate that the observed $\Delta S$ yielded negative values for all three thiols under the experimental conditions with a magnitude smaller than the absolute entropy of adsorption of ethanethiol in the gas phase (70.8 cal/mol·K).\textsuperscript{42} At 298.15 K, the $\Delta S$ indicates a non-linear behavior for the three different thiols tested (Table 5.3). This non-linearity stems from the lack of order in $C_2$ and $C_3$ thiols where van der Waals forces are probably minimal contributors. However, the larger decrease in $\Delta S$ for the $C_6$ thiol suggests a higher degree of ordering between adsorbed thiol chains occurs, and the associated van der Waals interactions lead to larger decreases in $\Delta S$, especially at low temperatures.

Another contribution to the observed $\Delta S$ values becoming more negative with decreasing temperature is the ordering of water molecules around the hydrophobic alkyl chains. Molecular dynamics (MD) simulations indicate that lower temperatures afford an
increased range of water structuring near a hydrophobic surface of an alkanethiol. The hydrophobic character of the thiol backbone makes it a structure maker; i.e., it leads to an increased ordering of water, an outcome of the hydrophobic effect. Recent advances in the structural characterization of water films near metal surfaces indicate water molecules on a metal surface have a well-defined two-layer structure. However, this structure is absent in the presence of an alkanethiol SAM on the metal surface. A consequence of the hydrophobic effect is the formation of cages of highly-structured water around the hydrophobic surface area of small molecule solutes (hydrophobic hydration); these structures are characterized by abnormally high heat capacity values. ITC can also provide information about solvation effects. By determining $\Delta H$ at a range of temperatures, the change in molar heat capacities ($\Delta C_p$) for a solute-solvent interaction can be determined from the slope of the linear regression analysis of observed $\Delta H$ vs. temperature. Our temperature-dependent ITC measurements (Table 5.3) are consistent with the expectation that $\Delta C_p$ increases as hydrophobic SAMs of increasing chain length are adsorbed on the Au NPs. This result suggests changes in the structure of the water surrounding the alkyl chains. In addition, the $\Delta C_p$ of all three thiol-NP complexes were found to be positive as a result of the exposure of the hydrophobic backbone to water. This further contributes to the observed negative $\Delta S$ values. Enzymes have multiple hydrophilic and hydrophobic surfaces exposed to water and provide reasonable comparisons with systems used in our study. Molar heat capacities of similar magnitude ($\Delta C_p = 1.5$ kcal/mol·K) have been observed in enzymatic studies and are attributed to the exposure of hydrophobic residues of proteins to water. As $\theta_{\text{thiol}}$ increases, a situation arises where both random and structured water are excluded from the interstitial space between thiol chains. MD simulations on the structure and dynamics of water
molecules confined between alkanethiolate monolayers on gold suggest that, at lower temperatures, the presence of water molecules near the alkyl chains of the thiols is more ordered than bulk water molecules and serves to decrease the entropy of the system.\textsuperscript{43,48} In addition, the COOH terminated thiols provide a hydrophilic surface and water molecules approach the hydrophilic surface more closely than a hydrophobic surface.\textsuperscript{45} Overall, the thiol-NP binding is enthalpically-driven and is accompanied by an entropic penalty associated with loss of translational and rotational degrees of freedom, and water molecules structuring near a hydrophobic surface. The current ITC study provides observed thermodynamic parameters and deconvolution of the effect of solvent requires a technique that allows one to probe the solvation environment around the NP surface. Ligand-receptor binding studies in H\textsubscript{2}O and D\textsubscript{2}O have shown that through successful coupling of ITC measurements with a Born-Haber thermodynamic analysis, the contributions of solvent effects to the thermodynamics of association can be deduced.\textsuperscript{49}

5.4.4. Influence of particle size on the thermodynamic parameters of binding of C\textsubscript{2}-TGA, C\textsubscript{3}-MPA, and C\textsubscript{6}-MHA to Au NPs

The three Au NP sizes used in this study are 5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm and Figure 5.6 represents the TEM images and size distribution of the Au NPs. To further quantify the adsorption thermodynamics at a thiol-NP interface, it is important to understand the dependence of adsorption parameters on NPs of various sizes. Table 5.5 indicates the thermodynamic parameters of adsorption of C\textsubscript{2}-TGA, C\textsubscript{3}-MPA, and C\textsubscript{6}-MHA on 5.4 ± 0.7, 9.5 ± 0.6 nm Au NPs, and C\textsubscript{3}-MPA, C\textsubscript{6}-MHA on 19.4 ± 1.1 nm Au NPs. The value of $\Delta H$ becomes more favorable with decreasing particle size for the three thiols tested. The Au NPs exposes mainly (111) terraces with a smaller fraction of (100) facets and under-coordinated
sites such as edges and corners. As NPs decrease in size, the number of under-coordinated sites relative to terrace sites increases. According to the theory on the statistics of surface atoms of perfect cuboctahedron NPs by Van Hardeveld and Hartog, the percentage of under-coordinated edges and corners for 5, 10, and 20 nm Au NPs are 11.3, 6.1, and 3.3% respectively. Adsorption onto these low-coordination sites is typically more exothermic than adsorption onto terrace sites. Therefore, with decreasing NP size, one would expect the initial $\Delta H$ to be more negative due to the presence of low-coordination defect sites. Table 5.5 indicates favorable changes in observed $\Delta H$ as a function of Au NP size with $C_2$, $C_3$, and $C_6$ thiol adsorption. The differences in the observed $\Delta H$ ($\Delta \Delta H$) for $C_2$, $C_3$, and $C_6$ thiols are 1.8, 3, and 11 kcal/mol respectively as the particle size changes from 5.4-19.4 nm ($\Delta \Delta H = \Delta H_{19.4nm} - \Delta H_{5.4nm}$). These differential ranges in $\Delta H$ indicates a secondary dependence on the alkyl chain length (in addition to a primary particle size dependence) due to other contributions, such as hydrogen bonding, and van der Waals interactions. In addition, the values of $\Delta \Delta H$ at varying NP sizes indicate that to a certain extent, the binding of thiols to different facets could influence the measured thermodynamic parameters, but precise determination of faceting effects is beyond the scope of this study.
Figure 5.6. (A-C) TEM Micrographs of Au NPs used in our study and (D-F) represent their corresponding particle size distribution. From the particle size distribution, the average diameter of the Au NPs use in our study are (A, D) 5.4 ± 0.7 nm, (B, E) 9.5 ± 0.6 nm, and (C, F) 19.4 ± 1.1 nm respectively. The total number of Au nanoparticles used to determine the size distribution histograms are 347 for 5.4 nm, 202 for 9.5 nm, and 217 for 19.4 nm respectively.
Table 5.5. Gold nanoparticle size dependence on the adsorption constant \( (K) \), Gibbs free energy of adsorption \( (\Delta G) \), enthalpy of adsorption \( (\Delta H) \), and the entropy of adsorption \( (T\Delta S) \) of C\textsubscript{2}-TGA, C\textsubscript{3}-MPA, and C\textsubscript{6}-MHA at 298.15 K.

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Au NP (nm)</th>
<th>( K ) (M(^{-1}))</th>
<th>( \Delta G ) (kcal/mol)</th>
<th>( \Delta H ) (kcal/mol)</th>
<th>( T\Delta S ) (kcal/mol)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsubscript{2}-TGA</td>
<td>5.4 ± 0.7</td>
<td>(5.1 ± 1.3) \times 10^6</td>
<td>-9.1 ± 1.1</td>
<td>-20.0 ± 1.3</td>
<td>-10.9 ± 1.2</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>9.5 ± 0.6</td>
<td>(3.9 ± 1.5) \times 10^6</td>
<td>-9.0 ± 1.3</td>
<td>-18.2 ± 1.6</td>
<td>-9.2 ± 1.4</td>
<td>0.27 ± 0.02</td>
</tr>
<tr>
<td>C\textsubscript{3}-MPA</td>
<td>5.4 ± 0.7</td>
<td>(3.3 ± 0.5) \times 10^6</td>
<td>-8.9 ± 0.4</td>
<td>-20.3 ± 1.1</td>
<td>-11.4 ± 0.4</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>9.5 ± 0.6</td>
<td>(8.4 ± 0.3) \times 10^6</td>
<td>-9.4 ± 0.5</td>
<td>-19.7 ± 0.1</td>
<td>-10.3 ± 0.5</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>19.4 ± 1.1</td>
<td>(4.5 ± 0.9) \times 10^6</td>
<td>-9.1 ± 0.1</td>
<td>-17.3 ± 0.3</td>
<td>-8.2 ± 0.1</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>C\textsubscript{6}-MHA</td>
<td>5.4 ± 0.7</td>
<td>(2.7 ± 0.4) \times 10^6</td>
<td>-8.8 ± 0.2</td>
<td>-28.6 ± 0.2</td>
<td>-19.8 ± 0.2</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>9.5 ± 0.6</td>
<td>(9.4 ± 0.7) \times 10^6</td>
<td>-9.5 ± 0.5</td>
<td>-24.7 ± 0.3</td>
<td>-15.2 ± 0.4</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>19.4 ± 1.1</td>
<td>(8.8 ± 0.8) \times 10^6</td>
<td>-9.4 ± 0.4</td>
<td>-17.9 ± 0.3</td>
<td>-8.5 ± 0.4</td>
<td>0.22 ± 0.01</td>
</tr>
</tbody>
</table>
The entropic contributions also show variation with changing particle size (Table 5.5). With decreasing particle size, the observed ΔS become more negative. This is indicative of that fact that thiol arrangement is more “solid-like” on the surface of smaller NPs. However, literature states that smaller NPs will allow for interactions between the tail groups and a greater freedom of movement of interlayer methylene groups which represents a higher packing disorder and hence a more “liquid-like” arrangement. In the case of larger NPs, there is an increase in the number of van der Waals interactions between the alkyl chains on the larger NPs; thereby creating a more “solid-like” arrangement. Similar behavior has previously been reported for long-chain alkanethiol adsorption on Au colloids and flat Au surfaces, where a “liquid-like” arrangement was observed on 2 nm Au colloids, and the packing disorder increased with decreasing alkyl chain length, and a “solid-like” arrangement was observed on flat Au surfaces. However, it is important to understand that the distinction between the packing of thiol ligands on Au surfaces has been characterized only for extremely well-defined SAMs formed from long-chain alkanethiols (n > 10). In this study, we are dealing with short-chain thiols which do not result in well-defined SAMs and hence the ability to clearly distinguish the packing orientation of thiols on Au NP surfaces based on the observed thermodynamic parameters is not appropriate. Furthermore, the values obtained from ITC are observed ΔS values and the exact interpretation of solid-like vs. liquid-like arrangement of adsorbed thiols on NPs of different sizes will be possible by evaluating the individual contributions to the observed ΔS values, which is beyond the scope of this current study.

5.4.5. Prevalence of enthalpy-entropy compensation in Au NP-thiol binding energetics
For all three Au NP sizes, irrespective of the thiol used, our data indicates a linear relationship between the change in enthalpy and entropy (Figures 5.7 and 5.8). This phenomenon, referred to as enthalpy-entropy compensation occurs in many chemical processes, including complex dissociation, solvation, and ligand-receptor interactions. The formation of Au alkanethiolates from a reaction mixture indicate that favorable changes in $\Delta H$ are compensated by unfavorable changes in $\Delta S$, resulting in very small changes in the overall free energy of adsorption with NP size. An increase in $\Delta H$ due to stronger binding may directly affect $\Delta S$ by the restriction of movement of the interacting molecules.
Figure 5.7. Experimental compensation plot for the enthalpy and entropy of adsorption for C₆-MHA adsorption on 5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm Au NPs at 298.15 K.
Figure 5.8. Experimental compensation plot for the enthalpy and entropy of adsorption changes for (■) C₃-MPA adsorption on 5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm Au NPs at 298.15 K.
5.5. Conclusions

In conclusion, we have provided a detailed analysis of the thermodynamic profiles at a solvated organic-inorganic interface by considering the binding of various carboxylic acid-terminated alkanethiols to Au NP surfaces. It was observed that $C_2$-TGA and $C_3$-MPA bind to a three-fold hollow site on the Au NPs, while $C_6$-MHA binding indicates steric hinderance of certain Au sites in the vicinity of the primary adsorption sites. For all three thiols tested, the magnitude of $\Delta H$ was found to linearly increase with increasing alkyl chain length, decreasing temperature, and decreasing Au NP size. Thiol-NP interactions are enthalpy-driven and are accompanied by an unfavorable entropic contribution mainly associated with the reduced translational (2D versus 3D), reduced alkyl chain rotation and hindered trans/gauche interconversion. The thermodynamic investigations presented in this paper not only reveal distinctive features of monolayer formation and interfacial energetics, but also has broader impacts on future studies. ITC studies on larger nanocrystals with well-defined surfaces (with one specific facet) and a mixture of surfaces can help one understand how molecular or polymeric entities influence the faceting of nanoparticles. From an experimental point-of-view, the particle number density (which is proportional to the number of surface atoms) must be sufficient such that adequate signal-to-noise heat flow data can be obtained. The use of complimentary calorimetric techniques opens possibilities to study a broad class of hybrid organic-inorganic composites to give insight into bond formation and ordering. Future studies will also include the construction of a Born-Haber cycle to deconvolute the intrinsic binding thermodynamics from the thermodynamic contributions of the solvent to the observed values, and also to quantify the effect different solvents have on binding equilibria. Another area of immense interest to nanostructures synthesis is to determine the magnitude
to which differences in the binding of small molecules lead to isotropic versus anisotropic growth.

5.6. References


Chapter 6

Conclusions and Future Work

6.1. Conclusions of the Dissertation

We examined the catalytic activity of 5.4, 9.5, and 19.4 nm Au nanoparticles for the liquid-phase reduction of resazurin (non-fluorescent) to resorufin (fluorescent) in the presence of a reductant (hydroxylamine) using a single-molecule fluorescence approach. The single-molecule measurements were cross-examined by suitable ensemble-level UV-Vis spectroscopic measurements. We demonstrated a unifying reaction mechanism based on Langmuir-Hinshelwood formalism, which assumed non-competitive adsorption between resazurin and NH$_2$OH due to their adsorption on distinct active sites on the Au surface and competitive adsorption between water and the reactants onto the same type of Au sites, with the formation of resorufin assumed to be the rate-determining step, fit the kinetic data well. The calculated rate parameters at the single-molecule level and the ensemble-level were found to be similar and the kinetic and thermodynamic consistency of the proposed reaction mechanism was examined. We observed distinct differences in the rates of reaction between the single-molecule level and the ensemble level; a feature we attributed to the influence of the solvent. We established that inhibition of Au surface sites by water molecules affects the reaction rate at the ensemble-level. The biased nature of single-molecule level measurements allowed the determination of rate parameters by negating the influence of the solvent. Additionally, the influence of solvent was further confirmed by carrying out single-molecule level and ensemble-level measurements in D$_2$O.
Catalysis at the single-molecule level demonstrated significant advantages over ensemble-level measurements, wherein one obtains kinetic parameters over many individual nanoparticles and the average value(s) of many nanoparticles. In particular, single-molecule measurements demonstrated individual Au nanoparticles possess variations in activity among individual catalytic turnover events. Furthermore, by monitoring the catalytic activity of several individual Au nanoparticles through temperature-dependent measurements, we reported the heterogeneity in distribution of rate constants, activation energy, and desorption energy. A key advantage of performing kinetics at the single-molecule level is that it allows one to delineate desorption from reaction, which is extremely difficult to deconvolute through ensemble-level measurements. This work demonstrates the highly dispersive nature of Au nanoparticles through temperature-dependent single-molecule measurements and highlights the significant advantages of single-molecule measurements over ensemble-level measurements.

Having identified that variations in activity exist among individual turnovers on a single Au nanoparticle, we next transitioned towards determining the possible origins of these observed activity fluctuations. Single-molecule level measurements on different sized Au nanoparticles (5.4, 9.5, and 19.4 nm) indicated that the resazurin reduction reaction is structure-insensitive. ReaxFF MD simulations and lack of correlations among individual catalytic turnover events confirmed that both intrinsic restructuring and catalysis-induced restructuring are not possible origins for the observed temporal variations in activity. We eliminated different types of active sites on an individual Au NP by utilizing carboxylic acid-terminated thiols of varying alkyl chain length. By varying the coverage of 3-mercaptopropionic acid (C₃-MPA) and correlating the observed rate to the percentage of
different active sites on the Au surface, we confirmed that thiols chromatographically titrate the Au sites from higher reactivity to lower reactivity. A lack of correlation among individual turnover events in the presence of thiols emphasized that, temporal variations in activity among individual turnovers on the same Au nanoparticle (dynamic variation) and variations in activity among different nanoparticles (static variation) is practically indistinguishable from one another, and originate due to the intrinsic reactivity of different type of active sites on the same nanoparticle. The evolution of distribution of $\tau_{\text{off}}$ and $\tau_{\text{on}}$ from single-exponential decay to Gaussian distribution with increasing thiol coverage confirmed that fluctuations in activity originated due to the intrinsic reactivity of the different types of active sites. We also identified that higher alkyl chain length thiols self-assemble on the Au surface in a highly ordered manner thereby resulting in a greater reduction in activity fluctuations. This work demonstrates that activity of an Au nanoparticle can be tailored and controlled by modifying the coverage of small-molecule adsorbates of varying affinity to the Au surface.

The last aspect we focused on at the single-molecule level was to understand the reorientation dynamics of single resorufin molecules generated on the surface of a 5.4 nm Au nanoparticle as a result of the reaction between resazurin and hydroxylamine. The individual catalytic turnover events in our single-molecule fluorescence trajectories revealed the presence of a slow rise in on-times (rise time, $\tau_{\text{rt}}$) and were detected using our experimental time resolution of 45 msec. We attributed the presence of $\tau_{\text{rt}}$ to the rotation dynamics of resorufin on the Au surface upon formation. The rise times between individual turnover events were determined to be completely uncorrelated. Additionally, there was no correlation between the rise time associated with one catalytic turnover event with the preceding waiting
time for reactant to diffuse and adsorb on the Au surface ($\tau_{\text{dark}}$). We determined an activation barrier of 4.1 kcal/mol associated with reorientation of the product molecule on the Au surface. Through DFT calculations, we determined the preferred orientation for resorufin molecule on Au (111) surface is with its molecular plane parallel to the Au surface. We also determined the fluorescence rate of resorufin as a function of molecular angle with respect to the Au surface. We identified a fluorescence enhancement (i.e., an increase in the fluorescence rate) of resorufin as the angle of the molecule with respect to the Au surface changed from 90° to 0°. We related the possible reorientation of the fluorescent resorufin molecule to its preferred orientation on the Au surface as the most probable source of the observed rise times.

In a separate study, we examined the thermodynamic adsorption profile at a solvated organic-inorganic interface ($K, \Delta G, \Delta H, \Delta S, n$) by following the binding and organization of carboxylic acid-terminated alkanethiols of varying chain lengths (C\text{2}, C\text{3}, and C\text{6}) to the surface of gold nanoparticles (NPs) (5.4 ± 0.7, 9.5 ± 0.6, and 19.4 ± 1.1 nm diameter) using isothermal titration calorimetry (ITC). We showed that the thermodynamic parameters support a mechanism of step-wise adsorption of thiols to the surface of Au NPs and secondary ordering of the thiols at the organic-inorganic interface. We observed that C\text{2}-TGA and C\text{3}-MPA bind to a three-fold hollow site on the Au NPs, while C\text{6}-MHA binding indicates steric hinderance of certain Au sites in the vicinity of the primary adsorption sites. The adsorption enthalpies were chain-length dependent; enthalpy values became more exothermic as longer chains were confined compensating for greater decreases in entropy with increasing chain length. We observed an apparent compensation effect: the negative $\Delta H$ is compensated by a negative $\Delta S$ as the thiols self-assemble on the Au NP surface.
comparison of the thermodynamic parameters indicated that thiol-Au NP association is enthalpy-driven because of the large, exothermic enthalpies accompanied by an unfavorable entropic contribution associated with confinement of alkyl chains, reduced trans-gauche interconversion, and the apparent ordering of solvent molecules around the hydrophobic organic thiols (hydrophobic effect).

6.2. Future Work

6.2.1. Spatial dependence of simultaneous turnovers on catalytic activity of Au microplates using high-resolution optical microscopy

The electronic structure and physical properties of a metal nanoparticle are markedly different from the corresponding bulk-like particles of the same metal, and therefore it should display distinct catalytic properties. The catalytic activity of a nanoparticle is largely controlled by the composition of the surface facets and sites, and each individual facets and sites differ in activity.¹ The precise determination of location of these active sites with the maximum catalytic activity is extremely important to better understand the connection between catalyst structure and catalytic chemistry, which leads to tailoring of the catalytic properties to the precise needs of a particular process. Advances in surface science of heterogeneous catalysis have focused on the catalytic activity of various metal sites for a variety of chemical transformations; classic examples include oxidation of CO by gold nanoparticles, and selective reduction of NO by iron oxide nanoparticles.²³ Modern synthetic methods, characterization techniques, and theoretical and computational approaches offer unique insights into reaction mechanisms and design of nanostructured catalytic materials for optimum activity. The ultimate goal is to replicate the unique catalytic properties of any
single nanoparticle into many kilograms of catalytic material, all of it exhibiting the same catalytic properties, for efficient utilization in research and industrial processes. However, it is extremely difficult to determine the exact surface structure of a nanoparticle, due to its extremely small size. The closest structure one can study to replicate the structure of a nanoparticle is that of a microplate. In order to differentiate the activity of various surface sites and facets, it is imperative to study individual catalytic turnover events \textit{in situ} on a single catalyst particle in a spatially resolved manner. The following section describes a high-resolution optical microscopy technique that can allow for spatial characterization of a single catalyst particle under reaction conditions, thereby allowing for precise identification of locations of catalytic turnover events. This should provide potential insight into improving the catalytic activity of metal catalysts and designing new ones with better efficiency.

Optical microscopy is a widely used imaging tool in biological research since it has a number of inherent advantages over electron microscopy and spectroscopy. These include the ability to specifically label a particular molecular species, the relatively fast time resolution, and the non-invasive nature of light which allows living samples to be studied with little perturbation. The spatial resolution in far-field optical microscopy is limited by the diffraction of light.\textsuperscript{4} The “diffraction limit” in light microscopy limits the smallest observable details in the image to 200 - 300 nm in the lateral dimensions and 500 - 800 nm in the axial dimension. This is extremely large when compared to the typical molecular length scales in cells, leaving many biological problems out of reach of optical microscopy. Heterogeneous catalysts on the other hand are even smaller when compared to biological cells and to study surface reactions on heterogeneous catalysts by optical microscopy is equally challenging. To overcome this diffraction limit, Rust et al.\textsuperscript{5} developed a high resolution microscopy
technique, stochastic optical reconstruction microscopy (STORM). STORM was developed in the biological community and uses photo-switchable fluorescent probes to temporally separate the otherwise spatially overlapping images of individual molecules, allowing the precise localization of individual fluorescent labels on the sample. A population of photoswitchable probes is turned off/on randomly allowing reconstruction of the cellular structure.\textsuperscript{6,7} The imaging process consists of many cycles during which fluorophores are activated, imaged, and deactivated. In each cycle only a subset of the fluorescent labels are switched on, such that each of the active fluorophores is optically resolvable from the rest. At single molecule detection abilities, if an isolated fluorophore is imaged, the photons forming the image are distributed as described by the point spread function (PSF) of the microscope for the chosen source.\textsuperscript{8} The PSF describes the apparent surface brightness profile of a point source. The center of this image can be determined with much higher precision than its width. This allows the position of these fluorophores to be determined with nanometer precision. The process is repeated to allow more molecules to be localized. A high resolution image can then be constructed from the measured position of these probes. The resolution of STORM is limited by the accuracy with which individual fluorescent molecules can be localized during an off-on cycle.

In a more recent study, Chen and co-workers employed the use of TIRF to understand the site-specific catalytic activity of triangular and hexagonal nanoplates (13.1 ± 0.7 nm thick) encapsulated in mesoporous silica (43 ± 8 nm thick).\textsuperscript{9} They attributed that the corner regions are the most active, followed by edge regions and then flat facets. They also identified that specific activity within a flat facet of a nanoplate showed a radial gradient in catalyzing the conversion of resazurin to resorufin. However, the notion of distinct reactive
sites with intrinsically different function (one for reaction and other for product docking after formation) has been previously been proposed by the same group on individual Au NPs.\textsuperscript{10,11}

We have proposed a reaction mechanism in Chapter 2 for the reduction of resazurin to resorufin which is derived from the notion that reactants bind in a non-competitive manner to the surface of Au NPs and the NP is capable of supporting simultaneous turnovers on its surface (Figure 6.1) possessing active sites with disparate $\tau_{on}$ times. Since the particle size is small (5.4 nm diameter), it is difficult to determine the location of these catalytic turnover events precisely.
Figure 6.1. Section of a turnover trajectory demonstrating multiple on-levels during the reduction of resazurin to resorufin on an individual 5.4 nm Au NP. Experimental conditions are 4 μM resazurin, 1 mM NH₂OH, and 25 °C.
However, by carrying out the same reaction of Au microplates, we can increase the probability of identifying simultaneous catalytic turnover events on the same Au particle located at a specific distance from each other and the correlation between the turnover events. We also believe that STORM can be achieved by using a simple fluorescent dye (resorufin) and a single-color continuously illuminating laser. The reduction of resazurin to resorufin on the surface of Au nanoparticles is naturally stochastic. We believe that by using this as the primary reaction on Au microparticles (0.5 – 10µm) and employing TIRF, we can identify individual fluorescent spots on the catalyst surface. The 520 nm green laser along with the flow cell accomplishes all three tasks required for STORM: exciting fluorescence from resorufin, deactivating it to the off state when the product diffuses away, and reactivating it to the fluorescent state.

We synthesized individual single-crystalline Au microplates (10 µm in lateral size) with regular shapes (triangular and hexagonal) via thermal reduction (80 – 150°C) of HAuCl₄ in the ethylene glycol in the presence of polyvinylpyrolidinone (PVP). This procedure was adapted from Kan et al.¹² (synthesized Au microplates of 10 µm in lateral size and 70 nm in thickness) and modified to suit our requirements. The procedure is described in detail in this section. 1 ml of HAuCl₄ (0.2 M in solution) was added to 6 ml ethylene glycol at 150 °C under continuous slow stirring conditions in a three-neck round-bottom flask. 3 ml ethylene glycol solution of poly(vinylpyrrolidone) (PVP) was injected drop wise under stirring conditions (the molar ratio of PVP/Au is 30). The reaction mixture was stirred continuously and after ~10 min, shining products appeared in the mixture and increased progressively with reaction time. Aliquots of the reaction sample was collected at 20, 40, 60, and 90 min. Due to the high boiling temperature of ethylene glycol (198 °C) and gelation properties of PVP, the
reaction mixtures aliquots were diluted with acetone (4× by volume) and centrifuged at 2500 rpm for 20 min, and the solvent containing residual reactants was decanted. The sample mixture was rinsed with deionized water and centrifuged ~ 3 times to remove all possible contamination. The samples were characterized using scanning electron microscopy (SEM). Figure 6.2(A-D) show the representative SEM images for Au microplates collected at different reaction times. The flat feature of the Au microplates is induced from their low and uniform contrast. The Au microplates exhibit a morphological transformation with increasing reaction times. At 20 min, the Au microplates are seen growing into triangular and hexagonal plates, but with not clearly defined boundaries (Figure 6.2A). The Au microplates sampled at 40 min show a mixture of truncated hexagonal, truncated triangular and regular triangular shapes (Figure 6.2B). At 60 min, the sample is dominated by thermodynamically stable hexagonal and triangular shapes with sharp corners (Figure 6.2C). The samples collected at 90 min showed distorted hexagonal and triangular Au microplates (Figure 6.2D). This SEM experiment is only an initial demonstration highlighting the possibility of synthesizing and characterizing Au microplates. It is necessary to follow up the SEM characterization by using atomic force microscopy (AFM) to define the shape and size of the synthesized Au microplates. Further characterization by x-ray diffraction (XRD) analysis will provide facet information on the Au microplates.
**Figure 6.2.** SEM images of Au microplates sampled at different reaction times: (A) 20 min, (B) 40 min, (C) 60 min, and (D) 90 min. These SEM images were obtained from Au microplates synthesized in our laboratory as an initial experimental trial.
As an initial experimental trial, we tried to perform the resazurin reduction on Au microplates (10 µm in lateral size) using single molecule TIRF as described in Chapter 2. A continuous-wave (CW) vertically-polarized (4-20 mW) 520 nm laser beam (35-KAP-431-220, Melles Griot) was focused onto a specific area in a microfluidic reactor cell (as described in Chapter 5) to directly excite the fluorescence of the product (resorufin) generated in situ on the immobilized Au microplates. An oil immersion objective (PlanAPO 60× /1.45 NA, TIRFM-2, WD 0.15 mm) was used for collection of the fluorescent photons. The slide and the coverslip was cleaned thoroughly using piranha solution before its assembly. The reaction solution consisted of 0.5 µM resazurin and 1 mM NH$_2$OH. We did not observe any reaction events when the microscope was operated in the “TIRF” mode. However, the presence of the Au microplate was confirmed by focusing the coverslip in the “wide-field” mode. An image of the hexagonal Au microplate focused in wide-field in represented in Figure 6.3. Previous work by Zhou et al. indicates that coating of the Au nanorods/microplates with a mesoporous silica shell aids in the detection of single reaction events.\textsuperscript{13} We believe that a similar modification to the Au microplate surface could aid in observing reaction events at the single-molecule level.
Figure 6.3. Wide-field image of a hexagonal Au microplate captured under constant flow of 0.5 μM resazurin and 1 mM NH₂OH. The “red” outline highlights the shape of the hexagonal Au microplate.
Due to their size, the Au microplates can be individually manipulated and deposited on glass slides and flow cells fabricated to facilitate the flow of the reactant solution (resazurin + NH$_2$OH). One easier way to detect the Au microplates under TIRF is to etch fiduciary marks on the glass cover slip using standard photolithography. These etch marks would serve as a guide to precisely locate the same Au microplate under TIRF conditions. This approach is similar to the one followed by Andoy et al. Multiple simultaneous turnover events can then be monitored at various locations on the microplates and their point spread function (PSF) can be determined. We aim to at least capture two turnover events happening simultaneously on the microparticle, where both of them are in the “on” state and one of them turns to “off” state (Figure 6.4). Using STORM, we aim to localize the individual reaction sites by determining the centroid position of the PSF of various bright spots. The obtained image can then be correlated with a corresponding SEM image of the Au microplate. The superimpositions of the SEM image with the image from the CCD camera will allow us to differentiate and quantify the catalytic activity directly at edges, corners and surface facets on an individual Au microparticle. In addition, the spatial arrangement of reaction sites should help us determine the effective distance between two reaction sites and the possibility of identifying a possible pattern in reaction sites of the surface-catalyzed reaction. We will extract individual $\tau_{off}$ and $\tau_{on}$ values and evaluate their autocorrelation function, which can help us ascertain the existence of a possible memory effect (i.e., turnover events happening on one active site influences the next turnover event happening at an adjacent site). Memory effect studies have been successfully demonstrated in single-molecule enzymatic studies which further describes the timescales between conformation fluctuations within a single protein molecule.$^{14-18}$ Since majority of the single turnovers on a
single microparticle will be asynchronous in time, this study would help us address the spatial heterogeneity present among surface atoms in addition to the temporal dynamics that is prevalent in nanoparticle catalysts.
Figure 6.4. STORM imaging sequence showing a hexagonal shaped gold microparticle with various reaction sites that can be switched from an “off” state to an “on” state due to the conversion of non-fluorescent resazurin to highly fluorescent resorufin by a green laser. All the hypothetical reaction sites are in the off-state (black dots) in the first image. In each imaging cycle, a 532 nm green laser pulse is used to randomly activate different active centres to the “on” state (red dots) as a result of the product being formed. Once the product is formed and desorbed from the Au microparticle surface, the reaction site switches back to the “off” state. After several imaging cycles, the position of each individual reaction site can be determined with high accuracy (red crosses). The overall image is then reconstructed from the reaction site positions obtained from multiple imaging cycles. The challenge here is to capture two independent “off-on” states simultaneously in order to determine two reaction sites and the distance between them.
6.2.2. Spatial and temporal differences in reaction selectivity at the single-molecule level

Metallic nanoparticles offer unique opportunities for catalysis primarily due to their high surface area. Reaction selectivity refers to the production of one product molecule out of many other thermodynamically feasible molecules, and distinctly measurable differences in potential energy barriers of single reaction steps control the yield of the desired product molecule (selectivity), while the overall activation energy controls the turnover rates (activity). Selectivity studies on colloidal nanoparticles and crystal surfaces have highlighted several molecular factors which influence selectivity in heterogeneous catalysts: adsorbate mobility, formation of reaction intermediates, adsorbate-induced restructuring, oxidation states, charge transport, surface structure, and surface composition. Understanding the kinetics of a catalytic process at nanoscale metal-liquid interfaces is crucial in determining reaction selectivity. This is typically carried out using macroscopic kinetic experiments; however, recent advances in optical microscopy provide an opportunity to probe the kinetics of a chemical process with in situ observations for single catalyst particles. Our overall objective is to acquire the knowledge to effectively engineer, manipulate, design, and control nanoparticle catalysts with superior catalytic properties for desired chemical transformations. This is possible only through a fundamental understanding of the structure-activity and structure-selectivity relationships. Through my PhD research, we have managed to address aspects of structure-activity relationships through single-molecule and ensemble-level measurements on Au nanoparticles. The next step towards our overall objective is to understand structure-selectivity relationships and to define the link between nanoparticle reactivity and reaction sensitivity/product composition. This can be addressed by single-molecule and ensemble level measurements by following a fluorescent off-on
reaction where a single non-fluorescent (off-state) reactant gives rise to two spectrally distinct fluorescent (on-state) products.

Boron dipyrrins are highly colored compounds that show intense fluorescence. The acylation of 2,4-dimethylpyrrole with acetic anhydride and boron trifluoride as Lewis acid catalyst resulted in the formation of a highly fluorescent compound (Figure 6.5). These compounds are based on the 4,4-difluoro-3a,4a-diaza-s-indacene core and are sold under its brand name of BODIPY. They display many highly desirable properties, such as high quantum yields of fluorescence, high extinction coefficient, and high photostability. Their absorbance and emission profiles tend to be relatively sharp and are only slightly Stokes shifted. They are uncharged, and their characteristics are mostly independent of solvent polarity. The BODIPY compounds possess low toxicity and are stable in physiological pH-range, only decomposing in strong acidic and basic conditions. These properties make them excellent probes for use in biological materials and novel materials. Through functionalization of the dye, it is possible to modify the physicochemical and spectral properties of the dye.
Figure 6.5. Synthesis of boron dipyrrins dye by acylation of 2,4–dimethylpyrrole (1), with acetic anhydride and boron trifluoride as Lewis acid catalyst. The dipyrrins (3) forms as a result of acid catalyzed condensation of (1) and acylated pyrroles (2). Complexation of (3) with a boron difluoride unit results in the fluorescence compound (4).
One of the first studies to monitor a catalytic reaction through the use of a BODIPY probe at the single-molecule level was by De Cremer et al.; they studied individual reactions based on a fluorogenic reporter.\textsuperscript{35} They utilized a phenylbutadienyl-substituted boron dipyrrromethene difluoride (PBD-bodipy) probe as a substrate for an epoxidation reaction on the surface of a Ti-MCM-41 particle. In the presence of tert-butylhydroperoxide (TBHP), the red fluorescent bodipy probe gets converted into two yellow emitting products and can be monitored using single-molecule fluorescence microscopic techniques. We will make use of the knowledge that epoxidation of PBD-bodipy results in two distinct fluorescent products and that Ag, Au, TiO$_2$, and Au-Ag bimetallic nanoparticles are well-known epoxidation catalysts. However, it is extremely difficult to probe in real-time the structure-sensitivity relationship of these metal catalysts during a chemical reaction. From our structure-activity studies on Au nanoparticles using single-molecule TIRF, we believe that the same technique can be used to probe the structure-sensitivity relationships of different metal catalysts. Towards this, we utilize Au nanoparticles to catalyze the epoxidation of PBD-bodipy into two fluorescent products that emit photons with a large enough Stokes shift and aim to observe their formation simultaneously in real-time using TIRF (Figure 6.6). These experiments would allow us to examine temporal differences in reaction selectivity. The spatial differences in reaction selectivity can be probed by carrying out the reaction on Au microplates similar to section 6.2.1. In addition, we will also probe the influence of surface structure, surface composition, adsorbate-induced restructuring and formation of reaction intermediates through pH-dependent and temperature-dependent measurements.
Figure 6.6. Epoxidation of PBD-bodipy by tert-butylhydroperoxide (TBHP) on Au nanoparticles. The figure represents an experimental schematic of a section of the flow reactor used to image catalytic turnover events on the surface of individual Au NPs. A glass cover slip is functionalized with 3-aminopropyltriethoxysilane (APTES) and citrate-stabilized Au nanoparticles are electrostatically-adsorbed onto this surface. The red fluorescent PBD-bodipy probe is epoxidized in the presence of TBHP on the Au active sites. Upon catalytic reaction, two yellow fluorescent products $P_1$ and $P_2$, distinctly different fluorescence emission wavelength are formed.
The synthesis of the PBD-bodipy probe is described in detail elsewhere.\textsuperscript{35,36} This section provides a brief description of the synthesis procedure. 2 g (21 mmol) of pyrrole-2-carboxaldehyde, 9.4 g (21 mmol) of cinnamyl triphenyl phosphonium chloride and 1.4 g of zinc dust are mixed and heated at 100°C for 15 h under nitrogen. After cooling to room temperature, the purple mixture is dispersed in 50 ml chloroform and filtrated. The solution is washed three times with water, suspended in chloroform, and dried with MgSO\textsubscript{4}. The product [phenylbuta-1,3-dien-1-yl]-1H-pyrrole was purified by silica chromatography with heptane:DCM (7:3) as eluens. To a mixture of 351 mg (1.8 mmol) of the purified product and 216 mg (1.8 mmol) of 3,5-dimethyl-pyrrole-2-carboxaldehyde in 90 mL dichloromethane, 180 μL (1.92 mmol) phosphorous oxychloride is added. This mixture is stirred for 18 h at room temperature under inert atmosphere. 1.32 mL (7.56 mmol) of diisopropyl-ethylamine, and 15 minutes later, 900 μL (7.32 mmol) of boron trifluoride etherate is added to the reaction mixture. After 1 h of stirring the reaction is complete. The mixture is washed two times with water, suspended in chloroform, and dried with MgSO\textsubscript{4}, followed by filtration and evaporation. The red phenylbutadienyl bodipy product is purified by silica chromatography (2.5 – 3 cm in diameter and 10 - 20 cm in height) with pure dichloromethane as eluens. The first fraction (RF = 0.95) is the desired product.

The formation of individual emissive product molecules can be carried out by interfacing a homemade flow cell (similar to one presented in Chapter 2 and 3) with TIRF microscope (see Chapter 2 for details on the microscope) and at 488 nm illumination. This will allow us to monitor the individual fluorescent bursts due to the production of a single product molecule. An organic solvent is typically used for these experiments; PBD-bodipy is known to be soluble in \textit{n}-butanol. The Au nanoparticles are electrostatically-adsorbed on an
APTES functionalized 40 mm round glass coverslip. The concentrations of the reactants are diluted (μM – nM) in order to bias the system to one catalytic turnover at any given point in time. We expect the single molecule trajectories to follow a similar pattern (off-on behavior) to the ones observed for the resazurin reduction to resorufin system (Chapter 2). By placing a dual emission splitter (Photometrics DV2, two-channel simultaneous-imaging system) in front of the CCD camera, it is possible to acquire two spatially identical but spectrally distinct images simultaneously. This will allow us to observe the formation of two product molecules in real-time from a single experiment. Movies of fluorescence bursts will be analyzed using a home-written MATLAB program, which extracts the individual fluorescence intensity trajectories from localized fluorescent spots for the entire time span of the movie. Further analysis pertaining to the statistical analysis of the individual off- and on-times will be carried out using home-written Mathematica codes.

At the ensemble-level, the reaction can be monitored using a stopped-flow spectrometer. The experimental method that can be employed is similar to the ones employed for temperature-dependent resazurin reduction reaction in Chapter 4. The only difference being the use of two different emission filters to filter out the photon emission from individual products. The fluorescence emission from the two products can be tracked simultaneously using two PMT detectors.

As an extension from my current PhD work, we believe that, the following aspects when effectively probed, can address the influence of kinetic dispersion on spatial and temporal selectivity at both the single-molecule and the ensemble-level.
Objective 1. Examine the influence of temperature, pH, and solvent on the kinetic parameters of PDB-bodipy epoxidation at the ensemble and single-molecule level

We have demonstrated through temperature-dependent ensemble and single-molecule level measurements on reduction of resazurin to resorufin on Au nanoparticles (Chapter 2) that the reactants adsorb in a non-competitive manner to the Au surface. In addition, the proposed reaction mechanism and the observed measurements also highlighted the influence of solvent on the observed reaction rates. At the ensemble level, the influence of temperature and pH on the reaction kinetics can be studied by spectroscopic (UV-Vis and stopped-flow) and calorimetric (ITC) measurements. The influence of pH can prove to be an interesting study as BODIPY molecules are known to be stable in physiological pH range.\(^{31}\) The variation in reaction specificity can be followed as a function of pH and could possibly provide insights necessary to corroborate solvent effects. In addition, ensemble-level kinetic measurements can also serve to support the kinetic results observed at the single-molecule level, validate a proposed reaction mechanism, and to understand the influence of solvent on the reaction kinetics. The thermodynamics of a catalytic cycle cannot be measured at the single nanoparticle level, but individual kinetic parameters can be determined by temperature-dependent measurements. We will make use of the experimental methodologies followed in Chapter 2 and we believe that changes in temperature and pH will influence product selectivity in the case of PBD-bodipy epoxidation and individual nanoparticles will exhibit a continuous distribution of kinetic parameters.

Objective 2. Investigate the relationship between site-specificity and reaction-specificity at the single-molecule level in the presence of adsorbates of varying affinity
The ability to control the number of active sites on a nanoparticle surface has strong implications in the study of heterogeneous catalysis, more specifically where metal nanoparticles are employed as catalysts. The presence of small molecule adsorbates which do not directly participate in the reaction will affect the restructuring of metal atoms. In Chapters 3 and 5, we have shown that thiols bind strongly to Au nanoparticles and results in the chromatographic titration of the most active sites on the nanoparticle surface. The absence of dynamic variation in the case of thiol-covered Au nanoparticles results in successive catalytic turnover events occurring on the same type of active sites on an individual nanoparticle. By carrying out the PBD-bodipy epoxidation reaction on thiol-covered Au nanoparticles, we can examine the relation between reactivity of a specific type of active site on reaction selectivity. We hypothesize that selectivity of the products should strongly depend on the type of active site that is responsible for its formation and can be correlated with the Au surface covered by strong adsorbates. The effect of small molecule adsorbates on the PBD-bodipy epoxidation reaction can also be examined at the ensemble-level using stopped-flow spectroscopy. The ability to simultaneously detect formation of both the products distinctly will allow for the quantification of the relationship between nanoparticle surface coverage and reaction selectivity, and can be compared with the results obtained at the single-molecule level.

Objective 3. Examine the influence of nanoparticle size and structure on the reaction selectivity at the single-molecule level

We have confirmed through single-molecule measurements on 5, 10, and 20 nm Au nanoparticles that the resazurin reduction reaction is structure-insensitive. In order to understand the influence of nanoparticle size on product selectivity for the epoxidation of
PBD-bodipy, we would like to utilize Au nanostructures ranging from clusters \((\text{Au}_n: n = 5 \text{ to } 20)\) to nanoparticles \((1-5 \text{ nm})\). \textit{We hypothesize that in the case of Au clusters, the stochastic arrangement of gold atoms between individual catalytic turnover events will preferentially influence the selectivity between the two products, while at larger nanoparticle sizes, the epoxidation reaction will be structure insensitive.} The thermodynamics of association of each individual reactant to the Au nanoparticle surface can be examined through ITC and the influence of Au cluster structural variation on observed kinetic dispersion can be probed by single-molecule experiments.

\textit{Objective 4. Examine the influence of polarization on fluorescence emission of the two products at the single-molecule level}

When a fluorophore is excited by polarized light its emitted light is also polarized. The emitted light can be depolarized by several phenomena including rotational diffusion of the fluorophore. The depolarization of the emitted light by rotational diffusion as a function of time reveals the average angular displacement of the fluorophore between the time of excitation to the time of emission. Thus, by monitoring the change of emitted polarized light from a fluorophore, the change in its rotational diffusion can be closely monitored. Polarized total internal reflection fluorescence microscopy can be used to detect the rotational dynamics of single molecules.\textsuperscript{37} It determines the extent of wobble of a fluorescent molecule bound to the macromolecule of interest. The rotational dynamics of a fluorescent molecule can be probed at the ensemble-level using polarization anisotropy measurements. By using a vertically polarized incident light, \textit{we hypothesize that changes in observed intensity between individual product formation events can be recorded by employing a polarizer at a specified...}
orientation to the emitted light and the results can be related to product rotational dynamics on the Au nanoparticle surface.

Objective 5. Understand the spatial differences in reaction specificity amongst individual catalytic turnovers on the surface of a microparticle during fluorescent two-state reactions using STORM methodology

STochastic Optical Reconstruction Microscopy (STORM) is currently emerging as a useful technique in imaging cellular structures; we would like to extend its application into heterogeneous catalysis and use it to quantify, within the resolution of STORM, the location of catalytic turnover on a microparticle. Since the PBD-bodipy epoxidation reaction is naturally stochastic, we hypothesize that simultaneous turnover events that are asynchronous in time but with some temporal overlap can be visualized and using STORM, enables us to determine the spatial differences in reaction selectivity amongst the locations of individual catalytic turnover events on the surface of the microparticle. Single turnover events are captured one at a time in the limit of infinite surface dilution of one of the reactants. The experimental procedure is similar to the one described in detail in Section 6.2.1 and the results would address the spatial dependence on reaction selectivity.

6.2.3. Reaction-based small molecule fluorescent probes for systematic analysis of chemical reactions

Most of the common industrial processes are based on bond cleavage reactions, organic addition and/or metal-ligand substitution reactions (Figure 6.7). The focus of my Ph.D. research was to understand the catalytic activity of Au nanoparticles in catalyzing a model system which highlights oxidation/reduction chemistry. In this section, we aim to
provide an account on different reaction chemistries that can be probed using fluorescent molecules at the single-molecule level. These reaction chemistries are widely prevalent in the industrial scale. The most common classes of fluorophores that can be used as probes for understand various catalytic chemistries include anthracenes, perylenes, BODIPYs, cyanines, rhodamines, fluoresceins, and oxazines (Figure 6.8). However, the most difficult phase during the study of reaction chemistries using fluorescent probes is the procurement, design, and linkage of the fluorescent reporters to the functional groups of interest. Most of the fluorescent probes are not available commercially and have to be designed and synthesized in the laboratory. This creates a multitude of challenges: the first is related to the multi-step synthesis steps which may require grams of several starting materials to achieve the desired yield of fluorescent probes. The second difficulty is in determining the functional groups that needs to be tethered to or may be intrinsic to the fluorescent probe for reaction compatibility during each step of the reaction. The third challenge lies in determining the suitable catalyst and the possible reaction sequence that is required to understand the reaction chemistry of interest. Recent studies have demonstrated multistep synthesis process for BODIPY and perylene classes of fluorescent probes to understand catalytic chemistry at the single-molecule level.35,39
Figure 6.7. Overview of the most common metal-NP mediated reaction-based strategies for the imaging of small fluorescent probes and metal-ion conjugates in heterogeneous catalysis. (A) Bond-cleavage reactions that occur on the surface of metal nanoparticles (NPs). These types of reactions include, but not limited to reduction, dehydrogenation, oxidative/reductive cleavage, carbon-carbon cleavage, and catalytic cracking reactions. (B) Organic addition and/or metal-ligand substitution reactions. The reactant and product molecules are generic “polyaromatic” shapes of a fluorescent probe and the green color on the product represents that the molecule is fluorescent.
Figure 6.8. A representative set of common classes of fluorophores that can be transformed into fluorescent probes that can potentially be used in chemical studies. R, alkyl group.
At the single-molecule level, it is identified that one of two different strategies towards using fluorescent probes is mostly employed. In the first type, fluorescent probes can act as participants in the reaction, i.e., the structure of the fluorophore chemically changes through bond formation or breaking at the fluorophore during the chemical reaction. In the second type, the fluorescent probes act as spectators, and the structure of the fluorophore does not change during the chemical reaction. Research progress on the type of chemical reactions that can be investigated using the single-molecule approach is still in the rudimentary phase. Participant probes have only been extensively studied at the single-molecule level for redox catalysis by Chen and co-workers, and for hydrolysis reactions by Roeffaers and co-workers. Spectator probes have been utilized to understand metal-ligand exchange reactions and polymerization reactions by Blum and co-workers. Oxidation, oxidation/reduction, oxidation/protonation, hydrolysis, metal-ligand exchange, and polymerization reactions represent the most common reaction mechanisms that involve fluorescent probes either as a participant or as a spectator and have been studied so far. We believe that all of these reactions are relevant to the metal NP catalyzed reactions prevalent in the chemical industries. Figure 6.9 represents a comprehensive list of the most common reaction chemistries that can potentially be applied to understand the catalytic activity of metal NPs under liquid-phase conditions. Recent developments in the field of single-molecule chemistry coupled with fluorescence microscopy techniques have demonstrated the applicability of the concept and the unique chemical information that can be accessed by these measurements. Exploring this avenue further will definitely provide the fundamental knowledge towards molecular level understanding of the catalytic activity of metal NPs. It
can also aid in improving the efficiency of the current catalysts as well as in designing new ones with optimized activity and selectivity.
**Figure 6.9.** Representative list of model chemical reactions catalyzed by metal NPs and involves small molecule fluorescent probes. Color on the product represents its emission color and no color indicates the “non-fluorescent” nature of the reactant molecule. (A) Oxidation reaction: the model reaction represents an epoxidation reaction that can potentially...
be carried out on the surface of either Au or Pt NPs and utilizes BODIPY molecule as the fluorescent probe. (B) Oxidation/reduction reaction: the reaction represents the conversion of non-fluorescent probe (amplex red) to the fluorescent molecule (resorufin) in the presence of an oxidant (H₂O₂), and on the surface of metal NPs. This reaction has previously been probed to understand the catalytic activity of Pt NPs. (C) Dehydrogenation reaction: The model reaction represents the conversion of non-fluorescent 3-(6-dimethylamino-2-naphthyl)-2-cyclohexenol into the fluorescent 3-(6-dimethylamino-2-naphthyl)-2-cyclohexenone. This probe has previously been utilized to study the fluctuations of an enzyme (17β-HSD-10) which is involved in Parkinson’s disease. The reaction represents a chemistry that is most common in several industrial reactions (conversion of an alcohol into a ketone). This reaction can also be studied on the surface of metal NPs (as Au, Pt, and Pd are well-known catalysts for dehydrogenation reaction) to understand its catalytic activity. (E) Hydrolysis reaction: The model reaction represents a hydrolysis reaction using fluorescein derivatives (fluorescein ester) as the fluorescent probe and can be carried out on the surface of metal NPs. The same reaction has been studied on the surface of double-layered hydroxide (Li⁺-Al³⁺) crystals and the intrinsic reactivity of different facets on the crystal has been reported. (E) Oxidation/Protonation reaction: The model reaction represents the protonation of a non-fluorescent probe (of the perylene family) into a fluorescent probe in the presence of acetic acid. This reaction chemistry also presents a model reaction that can be utilized to probe the catalytic activity of metal NPs.
6.3. References


Appendix A

Mathematica Code to Obtain Time versus Fluorescence Intensity Plots from Single Molecule Movies

The construction of the flow reactor and the experimental procedure to obtain a single molecule movie using a TIRF microscope are explained in detail in Chapter 2. Movies of fluorescence bursts were analyzed using a home-written MATHEMATICA program (provided below), which extracts the individual fluorescence intensity trajectories from localized fluorescent spots over the entire duration of the movie.

SetDirectory["path name"]

fps=22;

numFrames=Import["Single Molecule movie file name.avi","Frames"]//Length

imageList=Import["Single Molecule movie file name.avi","ImageList"];

rawTrajectory=Reap[Do[Sow[Mean[Flatten[ImageData[imageList[[i]]],2]]],{i,1,Length[imageList]}]][[2,1]];

maxValue=Max[rawTrajectory];

scaledTrajectory=rawTrajectory/Max[rawTrajectory];

scaledTrajectoryAndTime=Table[{i/22,scaledTrajectory[[i]]},{i,1,Length[scaledTrajectory]}];

thresholdPercent=.1;

minOnFrames=3;

minOffFrames=3;

progressToEndOfOff:=While[scaledTrajectory[[iterator]]<thresholdPercent&&iterator<(numFrames-minOnFrames),iterator+=1]

progressToEndOfOn:=While[scaledTrajectory[[iterator]]>=thresholdPercent,iterator+=1]
checkIfOn := (Select[# < thresholdPercent & !@scaledTrajectory[[iterator ;; iterator + minOnFrames - 1]], ##]/Length) > 0

checkIfOff := (Select[# > thresholdPercent & !@scaledTrajectory[[iterator ;; iterator + minOffFrames - 1]], ##]/Length) > 0

onOffs = Reap[iterator = 1; Sow[iterator, x]; While[iterator < (numFrames - Max[{minOnFrames, minOffFrames}]), progressToEndOfOff; If[checkIfOn, Sow[iterator - 1, x]; Sow[iterator, y]; While[True, progressToEndOfOn; If[checkIfOff, Sow[iterator - 1, y]; Sow[iterator, x]; Break[[], iterator += 1]]], iterator += 1]]] ; Sow[numFrames, x]; ][[2]];

sortedOnOffs = {Table[{onOffs[[1, i]], onOffs[[1, i + 1]]}, {i, 1, Length[onOffs[[1]]], 2}], Table[{onOffs[[2, i]], onOffs[[2, i + 1]]}, {i, 1, Length[onOffs[[2]]], 2}]];

offTimesRaw = Table[(sortedOnOffs[[1, i, 2]] - sortedOnOffs[[1, i, 1]] + 1)/22., {i, 1, Length[sortedOnOffs[[1]]]}];

onTimesRaw = Table[(sortedOnOffs[[2, i, 2]] - sortedOnOffs[[2, i, 1]] + 1)/22., {i, 1, Length[sortedOnOffs[[2]]]}];

offTimesFrequency = Module[{sorted, sortedWithoutDuplicates, iterator2}, sorted = Sort[offTimesRaw]; sortedWithoutDuplicates = DeleteDuplicates[sorted]; Reap[Do[Sow[sortedWithoutDuplicates[[i]], Count[sorted, sortedWithoutDuplicates[[i]]]], {i, 1, Length[sortedWithoutDuplicates]} ]][[2, 1]]];

onTimesFrequency = Module[{sorted, sortedWithoutDuplicates, iterator2}, sorted = Sort[onTimesRaw]; sortedWithoutDuplicates = DeleteDuplicates[sorted]; Reap[Do[Sow[sortedWithoutDuplicates[[i]], Count[sorted, sortedWithoutDuplicates[[i]]]], {i, 1, Length[sortedWithoutDuplicates]} ]][[2, 1]]];

ListPlot[offTimesFrequency]
ListPlot[onTimesFrequency]

offBinWidth = 0.1;
onBinWidth = 0.125;

offTimesFrequencyBinned = Table[{i, Select[offTimesRaw, # >= i && # < (i + offBinWidth)] // Length}, {i, 0, Max[offTimesFrequency[[All, 1]]], offBinWidth}];
onTimesFrequencyBinned = Table[{i, Select[onTimesRaw, # >= i && # < (i + onBinWidth)] // Length}, {i, 0, Max[onTimesFrequency[[All, 1]]], onBinWidth}];

Histogram[offTimesRaw]
Histogram[onTimesRaw]
Appendix B

Mathematica Code to Obtain Rise-times from Individual Catalytic Turnover Events at the Single Molecule Level

Movies of fluorescence bursts were analyzed using a home-written Mathematica program (Appendix A), which extracts the individual fluorescence intensity trajectories from localized fluorescent spots over the entire duration of the movie. The obtained individual fluorescence trajectories were further analyzed using a home-written Mathematica code (provided below), which determines rise times associated with each individual trajectory and calculates the distribution of the obtained rise times.

data=Import["File Path Name.CSV","CSV"];
zeroThreshold=5; (*This threshold defines a noise floor that will be ignored when splitting up the data*)
horizontalThreshold=1; (*This threshold is used when you split the data into individual turnovers*)

Split time versus fluorescence intensity data into individual catalytic turnover trajectories

nonZeros=Reap[Do[If[Max[data[[i-hORIZONTALThreshold;;i+horizontalThreshold,2]]]>zeroThreshold,Sow[i]],{i,1+horizontalThreshold,Length[data]-horizontalThreshold}][[2,1]];
iterator=1;turnoverListRaw=Reap[While[iterator<Length[nonZeros],Sow[Reap[Do[If[nonZeros[[i+1]]>nonZeros[[i+1]]-1],iterator+=1;Sow[nonZeros[[i]],iterator+=1;Break[]],{i,iterator,Length[nonZeros]-1}]]][[2,1]]];turnoverListRaw=Table[{First[turnoverListRaw[[i]]],Last[turnoverListRaw[[i]]]},
{i,1,Length[turnoverListRaw]}];
binWidth = 5; rawIntensityVersusTime = Table[Mean[data[[turnoverListRaw[[i, 1]] ;; turnoverListRaw[[i, 2]]]], {i, 1, Length[turnoverListRaw]}]; binnedIntensityVersusTime = Table[{i, Mean[rawIntensityVersusTime[[i - (binWidth - 1)/2 ;; i + (binWidth - 1)/2]]]}, {i, 1 + (binWidth - 1)/2, Length[rawIntensityVersusTime] - (binWidth - 1)/2, binWidth}];
ListPlot[binnedIntensityVersusTime, PlotStyle -> Red, FrameLabel -> {"Turnover Index", "Average Intensity"}, Frame -> True]

**Fitting the turnover data by using a triangle and a rectangle**

shapeFn := Piecewise[{{m*x + b, x1 <= x <= x2}, {m*x2 + b, x2 < x < x3}}]
residual := Sum[(singleTurnover[[i, 2]] - (shapeFn /. {x -> singleTurnover[[i, 1]]}))^2, {i, 1, Length[singleTurnover]}]

takeBasicTurnover[i] := Take[singleTurnover, i];
riseTimes = Reap[Do[singleTurnover = Take[singleTurnover, i]; Clear[m, b, x1, x2, x3]; While[singleTurnover[[1, 2]] <= 0, singleTurnover = Drop[singleTurnover, 1]]; While[Last[singleTurnover][[2]] <= 0, singleTurnover = Drop[singleTurnover, -1]]; x1 = First[singleTurnover][[1]]; x3 = Last[singleTurnover][[1]]; findMin = Reap[Do[x2 = singleTurnover[[i, 1]]; Clear[m, b]; {m, b} = {m, b} /. FindFit[Take[singleTurnover, i], m*x + b, {m, b}, x]; Sow[{i, residual}]], {i, 2, Length[singleTurnover]}]][[2, 1]]; residualMin = Position[findMin, Min[findMin[[All, 2]]]][[1, 1]] + 1; x2 = singleTurnover[[residualMin, 1]]; Clear[m, b]; {m, b} = {m, b} /. FindFit[Take[singleTurnover, residualMin], m*x + b, {m, b}, x]; Sow[{If[residualMin == 2, 0, (x2 - x1)], Show[ListPlot[singleTurnover, PlotRange -> All], Plot[shapeFn, {x, First[singleTurnover][[1]], Last[singleTurnover][[1]]}], PlotStyle -> Red, PlotRange -> All, Frame -> True]}], {i, 1, Length[turnoverList]}][[2, 1]];
riseTimeDist = riseTimes[[All, 1]];
Mean[riseTimeDist]

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(*Export"File Name.GIF",riseTimes[[All,2]]*)

stDev=StandardDeviation[riseTimeDist]

**Determination of bin width and plotting the distribution of rise-times**

binWidth=3.46*stDev*Length[turnoverList]^(-1/3)*1.;

numBins=Round[(Max[riseTimeDist]-Min[riseTimeDist])/binWidth,1];

plot1=Histogram[riseTimeDist,numBins]

histoListRaw=HistogramList[riseTimeDist,numBins];

histoList=Transpose[{Table[(histoListRaw[[1,i]]+histoListRaw[[1,i+1]])/2,{i,1,Length[histoListRaw[[1]]]-1}].histoListRaw[[2]]},{i,1,Length[histoListRaw[[1]]]}];

scaleFactor=1/Sum[(histoListRaw[[1,i]]-histoListRaw[[1,i-1]])*histoListRaw[[2,i-1]],{i,2,Length[histoListRaw[[1]]]}];

probDist=Table[{histoList[[i,1]],histoList[[i,2]]*scaleFactor},{i,1,Length[histoList]}];

plot2=ListPlot[probDist,PlotStyle→Red]
EDUCATION

The Pennsylvania State University, University Park, Pennsylvania, USA - (Aug 2009 - Present)
Doctor of Philosophy in Chemical Engineering, GPA 3.5 (expected Graduation - May 2014)

University at Buffalo, The State University of New York, Buffalo, New York, USA - (Aug 2007 - Sep 2009)
Master of Science in Chemical and Biological Engineering, GPA 3.7

Anna University, Chennai, India - (Jul 2003 - May 2007)
Bachelor of Technology in Chemical Engineering, GPA 4.0 (Valedictorian)

CONFERENCE PROCEEDINGS

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- International Precious Metals Institute (IPMI) – Outstanding Student Award for Excellence in Precious Metals Research, 2013
- The Pennsylvania State University – Department of Chemical Engineering “Student Safety Award”, 2013
- AIChE Chemical and Reaction Engineering Division student award, 2012
- Richard J. Kokes award recipient at the 22nd North American Catalysis Society, 2011