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Single-molecule fluorescence imaging of nanocatalytic processes†

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This tutorial review covers recent developments in using single-molecule fluorescence microscopy to study nanoscale catalysis. The single-molecule approach enables following catalytic and electrocatalytic reactions on nanocatalysts, including metal nanoparticles and carbon nanotubes, at single-reaction temporal resolution and nanometer spatial precision. Real-time, in situ, multiplexed measurements are readily achievable under ambient solution conditions. These studies provide unprecedented insights into catalytic mechanism, reactivity, selectivity, and dynamics in spite of the inhomogeneity and temporal variations of catalyst structures. Prospects, generality, and limitations of the single-molecule fluorescence approach for studying nanocatalysis are also discussed.

1. Introduction

Imagine you can see a single reactant molecule, landing on a catalyst particle, transform into a product molecule. Imagine you can watch these individual reactions continuously, in real time, and for as long as you would like. Imagine you can pinpoint, with molecular precision, the site where the reaction occurs on the catalyst particle. Imagine your observations are chemically specific, where different molecules appear in different colors, and you can observe reactions on many catalyst particles simultaneously. Imagine all these observations can be done in solution conditions using a bench-top optical microscope!

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Weilin Meng is currently an undergraduate at Cornell University studying chemistry and mathematics. His research in Prof. Peng Chen’s group involves the synthesis and catalytic study of palladium and platinum nanorods.
Such capabilities will enable answering many fundamental questions about nanoscale catalysts, such as those that catalyze energy conversion reactions in photovoltaic cells and fuel cells. They will allow us to quantify the chemical reactivity of catalysts with the highest sensitivity possible, and the inhomogeneity among catalyst particles, even if the catalyst may undergo structural changes during reactions. The knowledge obtained will guide us to improve the performance of existing catalysts and design new ones, both crucial for meeting society’s energy challenge.

Among many techniques scanning probe microscopy has arguably the highest spatial resolution for imaging single molecules. Atomic resolution, which naturally offers chemical specificity, is achievable. However, real-time, solution condition measurements are technically challenging and flat surfaces are necessary. Inherently the scanning approach cannot perform parallel observations of multiple molecules, although fast scanning rates offer a way toward it. Recent advances in scanning transmission X-ray microscopy (STXM) made it possible to scrutinize the structure and chemical nature of single catalyst particles in situ, but STXM does not yet have single-molecule sensitivity.

Our group has been working toward these capabilities through an alternative approach—single-molecule fluorescence microscopy. We visualize individual reactions on nanoscale catalysts by detecting a fluorescent product at the single-molecule level. This approach builds on the pioneering work in single-enzyme studies, and it has also been employed recently in studying micro- and nano-scale solid catalysts.

The single-molecule fluorescence approach offers many advantages in studying catalysis at the nanoscale: (1) real-time, single-reaction temporal resolution (i.e., the highest sensitivity possible), (2) single-particle (or sometimes single-reactive-site) resolution in imaging nanoscale catalysis, (3) tens of nanometer in spatial precision from super-resolution optical imaging, (4) chemical specificity from spectral selection of fluorescence, (5) multiplexed observation from wide-field imaging, (6) in situ measurements under ambient solution conditions from optical detection, and (7) continuous, steady-state reaction conditions via coupling to a flow cell.

The single-molecule fluorescence approach also overcomes a major obstacle in studying nanoscale catalysts: their structural inhomogeneity—for example, individual metal catalyst particles differ in structure (e.g., size and shape) from one to another and under reaction conditions often vary from time to time (so-called static and dynamic structural inhomogeneity, respectively). The real-time single-reaction resolution of the single-molecule approach allows studying behaviors of individual catalysts in real time in contrast to the ensemble-averaged measurements.

Our group has used the single-molecule fluorescence approach to study two types of nanoscale catalysts: metal nanoparticles and carbon nanotubes. For the first type, we have studied the chemical catalysis by Au nanoparticles at the single-particle, single-turnover resolution. For the second, we have studied the electrocatalysis by single-walled carbon nanotubes at the single-reactive-site, single-reaction resolution. Here we review the methodology and some of the results, highlight the relevant insights into the activity of the catalysts, and discuss the limitations and generality of the single-molecule approach as well as the scientific opportunities emerging from present progress. Part of the contents here overlap with our earlier reviews on related subjects but of different focuses. We also refer the readers to the reviews by Brauchle, Hofkens, and Majima in the same issue of this journal on other single-molecule studies of catalysts, as well as an earlier perspective article from us that reviews related single-nanoparticle and single-molecule electrochemistry studies. A recent general review by Weckhuysen et al. summarizes modern spatiotemporal studies of catalytic solids.

2. Catalysis by metal nanoparticles

Metal nanoparticles are important catalysts for petroleum processing and energy conversion reactions. Among a population of nanoparticles, there is always some structural dispersion: individual nanoparticles differ in size and shape. Moreover, owing to their nanometer dimension they have high surface energy; under catalysis each nanoparticle can undergo dynamic surface restructuring or even large shape changes. These particle- and time-dependent structural variations of nanoparticles necessitate studying them at the single-particle level in real time.

Single-nanoparticle catalysis at single-turnover resolution

Fig. 1A and B depict our single-molecule fluorescence approach to study catalysis on individual metal nanoparticles. We disperse and immobilize the nanoparticles on a quartz slide so the individual nanoparticles are separated by many surface are necessary. Inherently the scanning approach cannot perform parallel observations of multiple molecules, although fast scanning rates offer a way toward it. Recent advances in scanning transmission X-ray microscopy (STXM) made it possible to scrutinize the structure and chemical nature of single catalyst particles in situ, but STXM does not yet have single-molecule sensitivity.

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Single-nanoparticle catalysis at single-turnover resolution

Fig. 1A and B depict our single-molecule fluorescence approach to study catalysis on individual metal nanoparticles. We disperse and immobilize the nanoparticles on a quartz slide so the individual nanoparticles are separated by many
microns. We design a reaction, catalyzed by the nanoparticles, that converts a non-fluorescent substrate molecule to a fluorescent product molecule. With the substrate solution flowing above the nanoparticles, each catalytic reaction on a nanoparticle generates a fluorescent product. Under laser excitation each product formation event results in a burst of fluorescence signal that can be imaged readily using total internal reflection fluorescence microscopy, giving real-time single-reaction resolution detection of single nanoparticle catalysis. Because the reaction products are continuously generated, such experiments allow monitoring reactions on a single nanoparticle for extended time without the limit of fluorescence photobleaching. This single-molecule approach can differentiate the catalytic behaviors not only from one particle to another but also from one time point to another for each nanoparticle.

Our specific system is the Au nanoparticle-catalyzed reduction of resazurin to resorufin by NH₃OH in aqueous solutions (Fig. 1B). The highly fluorescent molecule resorufin is the target of single-molecule detection. Fig. 1C shows a typical fluorescence image from a real-time movie of catalysis by pseudo-spherical Au nanoparticles of 6.0 ± 1.7 nm in diameter. The discrete, localized bright spots, each of diffraction-limited size (FWHM ~400 nm), are the fluorescence signals of product molecules adsorbed on individual Au nanoparticles. In the movie, each of these bright spots “flashes”, like flashing stars in a dark sky, where each flash is one reaction (i.e., one turnover) catalyzed by one Au nanoparticle.

The typical fluorescence intensity versus time trajectory from one nanoparticle contains stochastic off–on bursts (Fig. 1D). The digital nature of the fluorescence trajectory and the consistent height of the on-level are characteristic of single-molecule behaviors. Each sudden intensity increase in the trajectory marks a product formation event on the nanoparticle; each sudden intensity decrease marks a product dissociation event from the nanoparticle; and each off–on cycle corresponds to a complete single turnover. (The photobleaching and photoblinking of the fluorescent product are two parallel pathways exist: one a substrate-assisted pathway, in which the nanoparticle binds a substrate first before the product leaves the nanoparticle surface (reaction ii and iii), and the other a direct dissociation pathway (reaction iv).

This kinetic mechanism is experimentally manifested by the substrate concentration ([S]) dependences of \( \langle \tau_{\text{off}} \rangle^{-1} \) and \( \langle \tau_{\text{on}} \rangle^{-1} \) (\( \langle \cdot \rangle \) denotes averaging), which represent the time-averaged single-particle rates of product formation and product dissociation, respectively. When averaged over many nanoparticles, both \( \langle \tau_{\text{off}} \rangle^{-1} \) and \( \langle \tau_{\text{on}} \rangle^{-1} \) show saturation kinetics (Fig. 2B and C), and their behaviors are quantitatively described by the following equations:2,3,7

\[
\langle \tau_{\text{off}} \rangle^{-1} = \frac{\gamma_{\text{eff}} K_1[S]}{1 + K_1[S]} \tag{1a}
\]

\[
\langle \tau_{\text{on}} \rangle^{-1} = \frac{k_2 K_2[S] + k_3}{1 + K_2[S]} \tag{1b}
\]

The kinetic parameters here are defined in Fig. 2A, and \( K_2 = k_1/(k_1 + k_2) \). When \([S] \rightarrow \infty, \langle \tau_{\text{off}} \rangle^{-1} \) equals \( \gamma_{\text{eff}} \) and \( \langle \tau_{\text{on}} \rangle^{-1} \) equals \( k_2 \), where \( \gamma_{\text{eff}} \) is the single-particle catalytic rate constant that represents the combined reactivity of all surface catalytic sites and \( k_2 \) is the product dissociation rate constant in the substrate-assisted pathway. It is worth noting that in ensemble-averaged experiments it is difficult to deconvolute the [S] dependence of the product dissociation kinetics from that of the catalytic product formation kinetics. Yet with single-nanoparticle measurements, the temporal resolution of \( \tau_{\text{off}} \) and \( \tau_{\text{on}} \) enables dissecting them cleanly.

The resolution of \( \tau_{\text{off}} \) and \( \tau_{\text{on}} \) also allowed us to probe the size-dependent kinetics of both the catalytic product formation reaction and the product dissociation reaction: Au nanoparticles of different sizes differ in the initial slopes and eventual saturation levels in the [S] dependences of their \( \langle \tau_{\text{off}} \rangle^{-1} \) and \( \langle \tau_{\text{on}} \rangle^{-1} \) (Fig. 2B and C). A number of kinetic parameters show clear size dependence (Fig. 2D–G), including \( K_1 \), the substrate adsorption equilibrium constant, \( \gamma_{\text{eff}}/A \), the catalytic rate constant per surface area (A), \( k_2 \), the product dissociation rate constant in the substrate-assisted pathway, and \( k_{1b} \), the rate constant for direct product dissociation.

These size-dependent kinetic parameters can be analyzed quantitatively using a classical thermodynamic model from Parmon and Murzin (Fig. 2D–G). This model approximates a metal nanoparticle as a continuous thermodynamic phase and attributes its size effect to changes in its chemical potential from surface tension.23,24 The changes in chemical potential are then translated to changes in reaction kinetics through the Bronsted-Polanyi relation. This treatment is applicable for particles containing more than ~100 atoms, for which their energy level splitting is much smaller than the thermal energy and the quantum size effect is negligible.25 For gold, this size regime encompasses particles of larger than ~2 nm in diameter. The Bronsted-Polanyi parameters extracted from fitting the data provide information on the transition states of both the catalytic product formation and the product dissociation reactions.6

Kinetic mechanism and size-dependent catalytic activity

In the fluorescence turnover trajectories (Fig. 1D), the temporal resolution of the two stochastic variables, \( \tau_{\text{off}} \) and \( \tau_{\text{on}} \), so-called waiting times, dissects the catalytic kinetics into two parts: \( \tau_{\text{off}} \) contains the kinetics of the catalytic product formation reaction, and \( \tau_{\text{on}} \) contains that of the product dissociation. Under saturating NH₃OH concentrations, we found that the catalytic product formation reaction follows a Langmuir-Hinshelwood mechanism: a Au nanoparticle catalyzes the conversion of the substrate resazurin to the product resorufin while maintaining a fast adsorption equilibrium of the substrate (Fig. 2A, reaction i). For product dissociation, two parallel pathways exist: one a substrate-assisted pathway, in which the nanoparticle binds a substrate first before the product leaves the nanoparticle surface (reaction ii and iii), and the other a direct dissociation pathway (reaction iv).
Size-dependent selectivity in parallel reaction pathways

In the kinetic mechanism (Fig. 2A), the product dissociation contains two parallel reaction pathways. For a single Au nanoparticle, it may prefer one pathway to the other or take the two pathways equally. This differential preference (i.e., selectivity) between parallel reaction pathways can be probed directly by examining the kinetics of a single Au nanoparticle.

Experimentally, the selectivity of a single Au nanoparticle in the product dissociation reactions is manifested by the [S] dependence of its $\gamma_{on}$ and $\gamma_{off}$ from eqn (1a) and (1b). (D, E, F, G, H) Thermodynamic analyses of the size dependences of kinetic parameters. Solid lines are fits from a classical thermodynamic model.© Copyright 2010 American Chemical Society.

![Fig. 2 Kinetic mechanism and size-dependent catalytic activity.](image)

(A) Schematic of the kinetic mechanism. Au$_n$: Au nanoparticle; S: resazurin; P: resorufin. Au$_n$-S$_n$ represents a Au nanoparticle having n adsorbed substrate molecules at adsorption equilibrium. The fluorescence state (on or off) of the nanoparticle is indicated at each reaction stage. $\gamma_{eff}$ is the total number of surface catalytic sites on one Au nanoparticle. $\theta$ is the fraction of catalytic sites that are occupied by substrates and equals $K_1[S]/(1 + K_1[S])$, where $K_1$ is the substrate adsorption equilibrium constant. (B, C) Substrate resazurin concentration titrations of $\gamma_{on}$ and $\gamma_{off}$ of 6.0 nm Au nanoparticles. Each data point is averaged over the turnover trajectories of many nanoparticles. Solid lines are fits with eqn (1a) and (1b). (D, E, F, G) Thermodynamic analyses of the size dependence of kinetic parameters. Solid lines are fits from a classical thermodynamic model.© Copyright 2010 American Chemical Society.

![Fig. 3 Size-dependent selectivity in parallel product dissociation pathways.](image)

(A) Resazurin concentration dependence of $\gamma_{on}$ and $\gamma_{off}$ from three 6.0 nm Au nanoparticles of type I, II, and III behaviors. Solid lines are fits with eqn (1b). (B) Size-dependent relative subpopulations of Type I, II, and III kinetic behaviors. Figure A adapted from Xu et al.© Copyright 2010 American Chemical Society.
measurements, in which type-I nanoparticles dominate and the direct dissociation pathway is unidentifiable (Fig. 2C).

Strikingly, the subpopulations shift among the three types of kinetic behaviors when the particle size changes: with increasing size, the subpopulations of type I and II behaviors decrease while that of type III increases (Fig. 3B). As type III particles have nearly equal reactivity in the two parallel product dissociation pathways \((i.e., k_2 \approx k_3)\), the increase in their subpopulation indicates that larger Au nanoparticles are less selective in the product dissociation reactions. Therefore, these subpopulation shifts demonstrate that tuning nanoparticle size can tune reaction selectivity besides tuning activity as described earlier.

**Coupling between dynamic surface restructuring and catalysis**

Due to their size, metal nanoparticles have high surface energy, and their surface structures are dynamic especially under catalysis where the constantly changing adsorbate–surface interactions can further induce the surface to reconstruct. This structural dynamics inevitably leads to dynamic activity fluctuations of a single nanoparticle. These activity fluctuations are challenging to observe in ensemble-averaged measurements, as they are asynchronous from one particle to another. Real-time single-nanoparticle measurements can directly probe these dynamic activity fluctuations, for example as manifested in the temporal variations in the rate of turnovers for a single 6.0 nm Au nanoparticle (Fig. 4A).

These dynamic activity fluctuations have contributions from the reaction rate changes of both the \(\tau_{\text{off}}\) reaction (catalytic product formation) and the \(\tau_{\text{on}}\) reaction (product dissociation). These two contributions can be evaluated separately for each nanoparticle by extracting out the sequences of individual \(\tau_{\text{off}}\) and \(\tau_{\text{on}}\) from the fluorescence turnover trajectory and analyzing their autocorrelation functions \(C_{\tau}(m) = (\Delta\tau(0)\Delta\tau(m))/(\Delta\tau^2)\). Here \(\tau\) is either \(\tau_{\text{off}}\) or \(\tau_{\text{on}}, m\) is the turnover index number in the sequences, and \(\Delta\tau(m) = \tau(m) - \langle \tau \rangle\). Fig. 4B shows an exemplary \(C_{\tau}(m)\) of a 6.0 nm Au nanoparticle. Its exponential decay behavior manifests the activity fluctuation in the catalytic product formation reaction. The time constant of this exponential decay gives the fluctuation correlation time, which is also the timescale of the underlying surface restructuring dynamics.

The catalysis-induced nature of the activity fluctuations and the underlying surface restructuring are directly supported by the positive correlation between the activity fluctuation rates \((i.e., the inverse of the fluctuation correlation times)\) and the rate of turnovers. For all three sizes of Au nanoparticles that we studied, the activity fluctuation rates increase with increasing rate of turnovers, following approximately linear correlations (Fig. 4C). Linear extrapolations to zero rate of turnovers result in positive intercepts, which give the approximate rates of spontaneous (as compared with catalysis-induced) surface restructuring dynamics for Au nanoparticles in an aqueous environment, corresponding to timescales of tens to hundreds of seconds. Furthermore, larger nanoparticles always have slower activity fluctuation rates, both at any rate of turnovers and at the extrapolated zero rate of turnovers. This indicates slower dynamic surface restructuring for larger nanoparticles, consistent with their surface atoms

![Fig. 4](image-url) **Surface-restructuring-coupled catalytic dynamics.** (A) Trajectory of rate of turnovers for a single 6.0 nm Au nanoparticle at a saturating substrate concentration. (B) Autocorrelation function of \(\tau_{\text{off}}\) derived from the same fluorescence turnover trajectory as that in A. Solid line is an exponential fit. (C) Dependence of the activity fluctuation rates (the inverse of fluctuation correlation times) of the \(\tau_{\text{off}}\) reaction on the rate of turnovers for three different sizes of Au nanoparticles. (D) Surface restructuring rate \(r\) dependence on the rate of turnovers \(v\) and the nanoparticle diameter \(d\). Dots are experimental data. The meshed surface is a fit with the thermodynamic model in the text. (E, F) Size dependence of the activation energy \(\Delta E_{\text{sp}}\) and the rate \(r_{\text{sp}}\) of the spontaneous dynamic surface restructuring of Au nanoparticles. The gray shades denote the approximate errors. Figures A, B adapted from Xu et al.\(^2\) Figures C–F adapted with permission from Zhou et al.\(^3\) Copyright 2010 American Chemical Society.
being more stable than those of smaller nanoparticles and being less prone to reconstruction (Fig. 4C).

We formulated a simple thermodynamic model to treat the catalysis- and size-dependent dynamic surface restructuring.\(^6\) This model assumes a linear coupling between \(r\), the rate of dynamic surface restructuring of a Au nanoparticle, and \(v\), its rate of turnovers, as observed experimentally (Fig. 4C). It also assumes that the coupling parameter is linearly proportional to \(r_{sp}\), the rate of spontaneous dynamic surface restructuring, because how readily catalysis can induce surface restructuring should be correlated with how readily spontaneous surface restructuring can occur. It then follows:

\[
r = r_{sp}(1 + qv) \tag{2}
\]

where \(q\) is a proportionality constant and the two terms in the parentheses represent the spontaneous and the catalysis-induced contributions to the overall dynamic surface restructuring, respectively. The rate of spontaneous dynamic surface restructuring, \(r_{sp}\), can be related to its activation energy \(\Delta E_{sp}\):

\[
r_{sp} = r^0_{sp}\exp(-\Delta E_{sp}/RT) = r^0_{sp}\exp(-\beta E(d)/RT) \tag{3}
\]

Here \(r^0_{sp}\) is the rate of spontaneous dynamic surface restructuring when thermal energy is much larger than the activation energy; \(\Delta E_{sp}\) is taken as proportional to the cohesive energy \(E(d)\) of the nanoparticle (\(\beta\) is a proportionality constant, \(d\) is the nanoparticle diameter), as surface restructuring involves breaking or partial breaking of the bonds between surface atoms. The cohesive energy \(E(d)\) scales approximately to \(1/d^{2.26,27}\). This simple model sufficiently describes the catalysis- and size-dependent dynamic surface restructuring of Au nanoparticles (Fig. 4D).

Using the fitted value of \(\beta\), we obtained estimates of \(\Delta E_{sp}\), the activation energy of spontaneous dynamic surface restructuring, at about a few kcal/mol across different sizes of Au nanoparticles (Fig. 4E). In the range of 6–15 nm diameters, the estimated spontaneous surface restructuring rate \(r_{sp}\) is \(~0.004-0.017\) s\(^{-1}\), corresponding to a timescale of 60–250 s (Fig. 4F). Although our model is simple and does not consider factors such as solvent effects and possible changes in the chemical nature of the particle surface during catalysis, the values of \(\Delta E_{sp}\) and \(r_{sp}\) here provide direct estimates of the metal surface atom energetics and structural dynamics, which are fundamentally important for heterogeneous catalysis in all size scales.

**Dynamic switching of surface catalytic behaviors**

Owing to adsorbate–surface interactions and possible adsorbate–adsorbate interactions, catalytic properties of solid surfaces can be dependent on the concentration of reactant molecules on the surface and in the reaction medium. This is especially true for nanoparticle catalysts, whose surface structures are more susceptible to influences from adsorbates. In studying 6.0 nm Au nanoparticles, we observed their \([S]\)-dependent surface catalytic behaviours, showing abrupt switching between a low and a high catalytic reactivity state when the substrate concentration changes.\(^4\)

We discovered this \([S]\)-dependent dynamic surface switching by analyzing the variances (Var) of individual \(t_{off}^{-1}\) and \(t_{on}^{-1}\) from each fluorescence turnover trajectory. These variances quantify the amplitudes of the time-dependent fluctuations of \(t_{off}^{-1}\) and \(t_{on}^{-1}\) of a single Au nanoparticle. At a low \([S]\), the two-dimensional histogram of Var(\(t_{off}^{-1}\)) and Var(\(t_{on}^{-1}\)) of many 6.0 nm Au nanoparticles shows a single population (type-a, Fig. 5A). With increasing \([S]\), part of the type-a population switches to another distinct population with larger Var(\(t_{off}^{-1}\)) and Var(\(t_{on}^{-1}\)) (type-b, Fig. 5B). With further increase of \([S]\), all type-a switches to type-b (Fig. 5C).

Analyzing the type-a and type-b populations separately show that type-a has lower catalytic reactivity with stronger substrate binding and type-b has higher reactivity with weaker substrate binding.\(^4\)

The absence of intermediate behaviors indicates that the two types of surface catalytic behaviors do not contribute simultaneously; for each Au nanoparticle, it can behave as either type-a or type-b and switches at a certain critical concentration. The switching concentration also varies largely from particle to particle, spanning a concentration range of two orders of magnitude (Fig. 5D), reflecting the inhomogeneity among individual 6.0 nm Au nanoparticles.

We do not yet know the molecular details of the dynamic switching of Au nanoparticle surface behaviors. It could come from surface reconstruction induced by substrate adsorption and catalysis, from adsorption reorientation of substrate molecule on the particle surface, or from substrate–substrate interactions. In any case, the underlying process must be

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Fig. 5  \([S]\)-dependent dynamic surface switching of 6.0 nm Au nanoparticles. (A, B, C) Two-dimensional histograms of the variances of \(t_{off}^{-1}\) and \(t_{on}^{-1}\) of individual 6.0 nm Au nanoparticles at different resazurin concentrations. (D) Population distribution (%) of the 6.0 nm Au nanoparticles across different type-a-to-b switching concentrations. Figures adapted from Xu et al.;\(^4\) Reproduced by permission of the PCCP Owner Societies.
cooperative to behave like a switch, *i.e.*, having high-order kinetics on substrate concentration. Intriguingly, we only observed this dynamic switching behavior on the 6.0 nm Au nanoparticles; the larger Au nanoparticles (*i.e.*, 9.1 and 13.7 nm) do not exhibit this switching behavior. This size dependence suggests that this dynamic switching of surface catalytic behaviors results at least in part from the nanometer dimension of these particles.

The dependence of surface catalytic behaviors on substrate concentration has strong implications in experimental studies of nanoparticle catalysts or heterogeneous catalysts in general. It makes imperative to study heterogeneous catalysis at conditions relevant to real applications. This message resonates with the so-called "pressure gap" in the surface science of heterogeneous catalysis.\(^{28,29}\) Ultrahigh vacuum studies, for which many powerful spectroscopic techniques are available to provide rich information on catalytic mechanisms, should be complemented with high pressure, high concentration studies (*e.g.*, in solution) to gain a full understanding of the catalytic properties.\(^{29-32}\) And for nanoparticles, single-particle resolution is necessary and single-turnover resolution is desired.

3. Electrocatalysis by carbon nanotubes

Carbon nanotubes, including single-walled carbon nanotubes (SWNTs), can electrocatalyze reactions that are of interest for energy applications,\(^ {33}\) for example the oxygen reduction reaction.\(^ {34}\) For preparing SWNTs, current methods produce a mixture of nanotubes of variable chirality, which leads to variable electronic properties (*i.e.*, metallic or semiconducting) among individual nanotubes and thus to their variable electrochemical and electrocatalytic properties. Obtaining monodisperse SWNTs remains a challenge, although increasingly better post-synthesis purification methods are being developed.\(^ {35}\) To circumvent this polydispersion problem, we have developed a single-molecule fluorescence approach to study the electrocatalysis of SWNTs at the single-reaction, single-reactive-site resolution.

**SWNT electrocatalysis at single-reaction resolution**

Fig. 6A shows our experimental scheme using an electrochemical flow cell and total internal reflection microscopy. Our electrochemical flow cell design is similar to that by Barbara and Bard in their single-molecule spectroelectrochemistry studies.\(^ {36,37}\) We disperse well-sonicated, diluted SWNT suspensions onto an ITO-coated quartz slide, so individual SWNTs are spatially well separated. The conductive ITO surface serves as the working electrode, which can effectively change the chemical potential of SWNTs because of their small quantum capacitance.\(^ {38-40}\) A Ag/AgCl reference electrode and a Pt counter electrode are also placed in the flow cell. Above the working electrode we flow a solution containing a reactant that can be electrocatalytically converted by the SWNTs into a fluorescent product. Each electrocatalytic reaction generates a fluorescent molecule, whose fluorescence can be readily detected at the single-molecule level under laser illumination. By detecting every product molecule, we visualize the electrocatalysis of SWNTs at the single-reaction resolution in real time.

Our specific fluorogenic reaction is SWNT-electrocatalyzed two-stage electro-reduction of the non-fluorescent molecule resazurin in aqueous solution (Fig. 6B).\(^ {41}\) The first stage reduces resazurin (S) irreversibly to resorufin (P), a highly fluorescent molecule. The second stage reduces resorufin to the nonfluorescent dihydroresorufin (PH\(_2\)) and is reversible. The fluorescence of P is the target of our single-molecule detection. Fig. 6C shows a typical fluorescence image from a real-time movie of SWNT electrocatalysis at a constant applied electrochemical potential (all potentials cited here are referenced to the Ag/AgCl electrode). The image shows discrete, localized fluorescence spots of diffraction-limited size (FWHM \(~\sim~\)400 nm) on the ITO working electrode. Each of these fluorescence spots flashes stochastically and *repetitively* in the movie.

The repetitiveness of these localized fluorescence bursts is clearer in the time trajectory of fluorescence intensity from one of these spots (Fig. 6D): the trajectory contains stochastic on-off bursts throughout the movie. The digital, two-state nature of these fluorescence trajectories verifies the single-molecule detection of the electrocatalysis, in which each burst results from a single P formation reaction.

**Super-resolution optical imaging of discrete reactive sites**

The SWNTs studied here have diameters of about 1–2 nm, but they can be many microns long.\(^ {42}\) Electrocatalysis can potentially occur at various places on a SWNT: the honeycomb-structured sidewall, the ends of the nanotube, or defect sites that can reside anywhere along the sidewall. In the
fluorescence electrocatalysis movies (Fig. 6C), the repetitive occurrence of fluorescence bursts at localized, diffraction-limited spots suggests that the reactions occur at discrete sites (e.g., defect sites or nanotube ends) rather than on the nanotube sidewall, which is continuous. The diffraction-limited resolution (~400 nm, Fig. 7A) is insufficient, though, to confirm the discreteness of these reactive sites, as the diameters of these SWNTs are merely 1–2 nm.

Single-molecule fluorescence detection offers a way to break the diffraction-limited resolution down to ~20 nm, via the so-called super-resolution optical imaging method.\(^{43-45}\) This method uses two features of single-molecule fluorescence detection. The first is the nanometer accuracy in localizing the center of the emission point spread function (PSF) of a single fluorescent molecule in a wide-field image, made possible by the large fluorescence photon counts (the localization accuracy \(\propto 1/\sqrt{\text{number of photons detected}}\).\(^{46-48}\) Fig. 7B shows the Gaussian fitting of the PSF of a single \(P\) molecule at a SWNT reactive site; the molecule is localized to about \(\pm 4.5\) nm accuracy, even though the FWHM of this PSF is ~410 nm.

The second feature is the temporal separation of fluorescence detection of individual molecules that reside within the diffraction-limited resolution. This temporal separation is manifested by the off--on signals in the fluorescence intensity trajectories and can be achieved through either photo-induced switching of fluorescent molecules or electrocatalytic generation of individual fluorescent \(P\) molecules as is the case here (Fig. 6D). Consequently, the localizations of the many \(P\) molecules from a single reactive site can be determined individually, each down to an accuracy of a few nanometers.

The localizations of all \(P\) molecules can then be overlaid together (Fig. 7C), from which a two-dimensional histogram can be generated (Fig. 7D). This histogram follows a Gaussian distribution,\(^{44}\) whose FWHM gives a spatial resolution of ~20 nm, an order of magnitude higher than the diffraction-limited resolution (~400 nm) (Fig. 7A). This ~20 nm resolution is not yet close to the nanometer accuracy in localizing the center of a PSF (Fig. 7B), likely due to the orientational motions of the molecules.\(^{49}\)

Considering the diameters of these SWNTs are about 1–2 nm,\(^{42}\) the ~20 nm resolution here limits the dimension of SWNT reactive sites to be no larger than ~2×20 nm\(^2\). Although without experimental proof, we think these reactive sites are probably smaller than our resolution, i.e., at about the molecular scale. The nanometer dimension of these reactive sites offers a distinctive advantage: Each reactive site on a SWNT acts as an ultrasmall electrode, for which the mass transport is efficient,\(^{50}\) thus allowing the study of electrocatalytic electron-transfer kinetics at low reactant concentrations (see later).

**Reaction mechanism and reactivity**

In the fluorescence electrocatalysis trajectories (Fig. 6D), each sudden intensity increase corresponds to an electrocatalytic formation of a \(P\) molecule at a reactive site. The sudden intensity decreases primarily result from electro-reduction events of \(P\) to \(PH_2\)—other possible causes, such as \(P\) photobleaching, photoblinking, and dissociation from the SWNTs, all happen at much slower timescales (seconds to tens of seconds) and do not contribute significantly to these sudden intensity decreases.\(^{8}\)

Therefore, the single-reaction resolution of the fluorescence trajectories dissects the electrocatalytic kinetics into the \(\tau_{on}\) and \(\tau_{off}\) processes: \(\tau_{on}\) is the waiting time for electro-reduction of \(P\) to \(PH_2\) after a \(P\) molecule is formed at a reactive site; \(\tau_{off}\) is the waiting time for \(P\) formation and contains the binding of \(S\) to the reactive site from the solution; and their statistical properties report the kinetic mechanism of the associated electrocatalytic reactions. At any applied electrochemical potential, we found that the electro-reduction of \(P\) to \(PH_2\) contained in \(\tau_{on}\) follows a simple, one step \(P \rightarrow PH_2\) reaction kinetics (reaction \(i\), Fig. 8A). For the electrocatalytic formation of \(P\) contained in \(\tau_{off}\), two parallel reaction pathways exist: one involves a substitution reaction of \(PH_2\) by \(S\) at the reactive site followed by electrocatalytic reduction of \(S\) to \(P\) (reactions \(i\) and \(ii\)); the other is a direct electro-oxidation of \(PH_2\) to \(P\) (reaction \(iii\)), which is possible because \(P \rightleftharpoons PH_2\) redox is reversible (Fig. 6B).

This kinetic mechanism is manifested experimentally by the \([S]\) dependences of \((\tau_{on})^{-1}\) and \((\tau_{off})^{-1}\), which represent the time-averaged \(P\) reduction rate and the \(P\) formation rate at a single reactive site, respectively. At any electrochemical potential, \((\tau_{on})^{-1}\) is independent of \([S]\) (Fig. 8B and C, top). In contrast, depending on the applied electrochemical potential, \((\tau_{off})^{-1}\) shows either asymptotic increase or asymptotic decrease with increasing \([S]\) (Fig. 8B and C, bottom).
These behaviors are quantitatively described by the following equations:

\[
\langle \tau_{on} \rangle^{-1} = k_{4}^{\text{red}} \quad (4a) \\
\langle \tau_{off} \rangle^{-1} = \frac{k_{2}^{\text{red}}(k_{3}^{\text{ox}} + k_{1}[S])}{k_{3}^{\text{ox}} + k_{1}[S]} \quad (4b)
\]

The rate constants here are defined in Fig. 8A. As \(k_{4}^{\text{red}}\) is the rate constant of \(P \rightarrow \text{PH}_2\) electro-reduction, \(\langle \tau_{on} \rangle^{-1}\) is independent of [S]. For \(\langle \tau_{off} \rangle^{-1}\), when [S] → 0, it equals \(k_{3}^{\text{ox}}\), the rate constant of electro-oxidation of \(\text{PH}_2\) to \(P\) (reaction iii); when [S] → ∞, it approaches \(k_{2}^{\text{red}}\), the rate constant of electro-reduction of S to P (reaction ii). At different electrochemical potentials, \(k_{2}^{\text{red}}\) and \(k_{3}^{\text{ox}}\) have different relative magnitudes, leading to variable [S] dependence of \(\langle \tau_{off} \rangle^{-1}\). At more negative potentials, electro-reduction is more favorable (i.e., \(k_{3}^{\text{ox}} < k_{2}^{\text{red}}\)) and \(\langle \tau_{off} \rangle^{-1}\) increases asymptotically with increasing [S] (Fig. 8B, bottom); at less negative potentials, oxidation is more favorable (i.e., \(k_{3}^{\text{ox}} > k_{2}^{\text{red}}\)), and \(\langle \tau_{off} \rangle^{-1}\) decreases asymptotically (Fig. 8G, bottom).

For each SWNT reactive site, eqn (4a) and (b) allow the determination of its kinetic rate constants, which quantify the site’s reactivity in the associated reactions. Distributions of these rate constants can then be built among the SWNT reactive sites (Fig. 8D); the broad widths of these distributions directly reflect their reactivity inhomogeneity, which likely comes from the different electronic properties of individual nanotubes and which is masked in ensemble-averaged measurements.

**Interfacial electron transfer**

In the SWNT electrocatalysis studied here, both the \(\tau_{on}\) and \(\tau_{off}\) reactions involve interfacial electron transfer from a SWNT to a bound molecule sitting at the SWNT–solution interface. Interfacial electron transfer is a fundamental process for energy production, conversion, and storage. The single-molecule measurements of SWNT electrocatalysis offer unique opportunities to probe it: the single-reactive-site resolution removes the inhomogeneity from different nanotubes and reactive sites; the single-reaction resolution enables examining electron transfer cleanly without the convolution of hole transfer; the latter is especially advantageous as compared with electrochemical current based measurements, in which the current has contributions from both electron and hole transfer processes.

Considering the \(\tau_{on}\) reaction as an example, it is a single step electro-reduction reaction, \(P \rightarrow \text{PH}_2\), with \(\langle \tau_{on} \rangle^{-1}\) equal to \(k_{4}^{\text{red}}\), the rate constant of this reaction. By studying electrocatalysis across a range of applied electrochemical potentials, we can study how the electrochemical driving force changes the interfacial electron transfer kinetics. Fig. 9 shows our preliminary result of an electrochemical potential titration of \(\langle \tau_{on} \rangle^{-1}\) of a single SWNT reactive site. With increasingly negative potential, i.e., increasing driving force for electro-reduction, \(\langle \tau_{on} \rangle^{-1}\) increases and eventually saturates. This behavior is consistent with the theoretical simulations of the interfacial electron transfer kinetics for an electro-reduction reaction on a SWNT. Quantitative analysis of this type of data using the Gerischer-Marcus model can provide fundamental knowledge on the electronic coupling between the nanotube and the molecule, the reorganization energy, and the optimal rate for interfacial electron transfer.

**4. Prospects and limitations of single-molecule fluorescence approach to nanocatalysis**

The single-molecule fluorescence approach offers rich insights into the catalytic and electrocatalytic properties of nanoscale catalysts. Inhomogeneity among the catalysts can be overcome,
and individuality can be quantified. Temporal behaviors can be followed in real time to reveal intricate catalytic dynamics. As long as suitable fluorogenic probe reactions are available, any catalyst can be studied. The present progress raises many new scientific opportunities in heterogeneous catalysis as well. First is the nanometer spatial resolution inherent to single-molecule detection via the super-resolution imaging method. We have used it to resolve the discrete reactive sites on SWNTs, as discussed in section 3; others have used it to study zeolite catalysts.\textsuperscript{54,55} For nanoparticle catalysts, this nanometer resolution may resolve catalysis spatially on a single nanoparticle, thus offering insight into facet-specific information of surface reactivity. For carbon nanotubes, the location of reactive sites on the nanotube can be correlated to its reactivity. Coupling to parallel structural measurements on the nanocatalysts, using TEM or AFM for example, represents another big opportunity. The structure of a nanoparticle, or the diameter of the nanotube, can be correlated with the catalyst’s reactivity to generate unprecedented insights into structure-reactivity correlations. Our previous reviews contain more discussions on compelling scientific questions and opportunities.\textsuperscript{5,9}

Nevertheless, the power of the single-molecule approach does not come without limitations. As this approach is based on fluorescence detection, one of the reaction products needs to be fluorescent. Reactions that merely involve small molecules, such as methanol oxidation or CO\textsubscript{2} reduction, cannot be studied directly. The types of chemical transformations to be studied are not limited, however, as one can create reactant molecules that undergo the desired transformation to generate a fluorescent molecule. For example, if dehydrogenation reaction of C–OH bond is of interest, one can design a molecule that if dehydrogenated, the resulted C=O bond completes an extended conjugation, forming a fluorescent product. The probe reactions discussed in this review are examples as well: The resazurin to resorufin conversion involves cleavage of an N–O bond, a reaction of much interest. The resorufin to dihydroresorufin electro-conversion is an electron-coupled proton transfer reaction, involving two electrons and two protons, and is fundamentally related to the proton reduction reaction for fuel generation. Applying creative synthetic chemistry to generate suitable fluorogenic probes, many nanoscale catalysts can be studied.

As aromatic, fluorescent molecules are large and typically nonvolatile, this limits the study to reactions in solution, in which the large molecules can be soluble. Additional steric effects may exist in their chemical transformations; they can affect the reaction kinetics in a way not present in the chemistry of small molecules. Nonetheless, these steric effects can well be relevant and of interest on their own, as many catalytic reactions in industry are on large molecules. Even though most fluorescence microscopy experiments are done under ambient conditions, high temperature liquid phase reactions are also possible using specially designed sample chambers and a long working-distance air objective to avoid direct contact with the sample.

Single-molecule fluorescence measurements, although powerful in dissecting kinetic mechanisms of catalytic reactions, are less capable of determining molecular mechanisms, \textit{i.e.}, how chemical bonds are broken or made. This is partly due to their limited time resolution, \(\mu s\) at best, which cannot resolve the actual chemical transformations that occur at sub-picosecond timescales. Another reason is the limited chemical information contained in fluorescence; single-molecule vibrational spectroscopy \textit{via} surface enhanced Raman scattering (SERS) can be a complementary and powerful approach.\textsuperscript{56–61} In combination with other detection methods, such as electrical and mechanical measurements, single-molecule fluorescence approach can offer a myriad of possibilities to study nanoscale catalysis.

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