Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 11075–11076, September 1999
From the Academy

This paper is a summary of a session presented at the first Chinese-American Frontiers of Science symposium, held August 28–30, 1998, at the Arnold and Mabel Beckman Center of the National Academies of Sciences and Engineering in Irvine, CA.

Single molecule physics and chemistry

CHUNLI BAI*, CHEN WANG*, X. SUNNEY XIE†, AND PETER G. WOLYNES‡

*Institute of Chemistry, The Chinese Academy of Sciences, Beijing 100080, China; †Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 01238; and ‡School of Chemical Sciences, University of Illinois, Urbana, IL 61801

ABSTRACT New experiments using scanning probe microcopies and advanced optical methods allow us to study molecules as individuals, not just as populations. The findings of these studies not only include the confirmation of results expected from studies of bulk matter, but also give substantially new information concerning the complexity of biomolecules or molecules in a structured environment. The technique lays the groundwork for achieving the control of an individual molecule's motion. Ultimately, this work may lead to such practical applications as miniaturized sensors.

It is said that, when Richard Feynman was bothered while looking through one of the first scanning tunneling microscopes, he said he was upset to be interrupted because seeing the images of single atoms was a "religious experience". For many generations of scientists, the molecule was both the concrete, ultimate entity on which our understanding of the everyday world was based and an elusive intellectual construct whose very existence could only be inferred circumstantially by experiments on macroscopic samples. Seeing an individual atom or molecule going through its motions brings immediate, emotional impact to this central concept of modern thought. Recently, advancing technology has allowed experiments on individual molecules to open up new vistas on the molecular world. This raises the question, "When is molecular individuality important?" Truly elementary objects behave in statistically identical ways: All spin up positrons are alike, as far as we can tell. Thus, although one Academy publication was entitled *A Positron Named Priscilla*, (1), highlighting an experiment that trapped a single positron, there would seem little point in giving this particle a name. In particle physics, by using Poisson statistics, we can infer the behavior of a large population of unstable elementary particles after seeing only one (or a few) decay events. The same statistics apply to the simplest chemical transformations that involve isolated small molecules. This would suggest single molecule experiments would have little to offer to chemistry. Nevertheless, the new possibility of studying single molecules is important because molecular individuality does finally come into play when the molecule is a complex entity. This may occur because the molecule itself may have an intricate internal structure—e.g., a biomolecule, resulting in a complex energy landscape. Alternatively, the molecule may be part of a complex environment that substantially changes its behavior. Here, distinguishing different molecules at different locales is crucial for understanding the system as a whole. Biomolecules in living cells are such an example. Even simple inorganic molecules on structured surfaces or in disordered systems such as viscous liquids or glasses provide situations in which molecular individuality matters. In all of these cases, the capability of studying an individual molecule over time can give new insights unavailable by straightforward experiments on macroscopic populations of molecules. The new questions that single molecule experiments let us ask move chemistry and physics into a realm more familiar to the astronomers who have direct observational knowledge of a single complex object (the universe) and must infer the underlying rules and patterns from that example.

Two techniques were discussed that give different perspectives on single molecule behavior. In our symposium, we compared the advances made by studying single molecules bound to surfaces studied by scanning probe microscopy (SPM), and the behaviors of single molecules in solutions or within enzymes, addressed using fluorescence microscopy.

With the aid of SPM, direct observations of entire arrays of atoms, molecules, and the fine structures of molecular aggregates have become possible (2–5). For example, Fig. 1 shows the atomic force microscopy image of a molecular globule of polycation-DNA complex (3). The capability of precisely controlling probes allows us to study the long-range structures made by molecules laying on surfaces. Although pretty pictures are easy to make, obtaining quantitative characteristics of surface bound molecules is not entirely straightforward. Rigorously interpreting SPM images requires substantial theoretical as well as experimental effort.

SPM technique has been actively used to search for many different signatures of the presence of atoms and molecules. Feeling the presence of atoms by the forces they exert on a standing tip is one approach used in atomic force microscopy. Electron tunneling and spectroscopy are more subtle probes. Magnetic and optical sensing, in conjunction with SPM technique, also prove to be very illuminating. It is interesting to note that it was once debated whether the laws of quantum mechanics allowed a single free spin to be detected. Having a precise spectroscopic signature for each molecule is crucial for identifying the individual species. Without such signatures, it is hard to compare with the highly precise data available for bulk samples. An example of such an insightful spectroscopic probe is the recent use of vibrational species to identify the stretching mode of a single acetylene molecule and surface. (6).

The ideal probe must not only be atomically sharp but also chemically and physically specific. There are a large number of candidates for such probes under exploration. Carbon nanotubes are certainly sharp enough and act as superb probes once attached to a normal tip apex (7–9). These probes become even more versatile through chemical modification (9). Treatment with various thiols and surfactants produce tips with known species (chemically or electrically) at their apex. The different interactions between these probes with the substrate potentially give great precision to a chemical map of a sample's surface.

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Abbreviation: SPM, scanning probe microscopy.

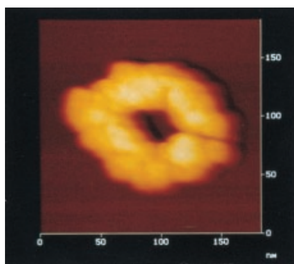

FIG. 1. Atomic force microscopy image showing the fine structure of a monomolecular toroidal globule of λ-DNA (48 kbp, a full length of ≈17 μm) (adapted from ref. 3).

Recent advances in microscopy have allowed optical detection of individual molecules even when they cannot be imaged very well. Unlike scanning, optical techniques can currently provide the high time resolution necessary for dynamical studies. This makes up for their lesser spatial resolution in some ways. Near-field microscopy is capable of imaging single molecules with a spatial resolution beyond the diffraction limit (10), but conventional optical microscopes can be more easily used to study the time behavior of single molecules for very dilute samples. The most widely used approach is fluorescence detection. This sensitive method was first applied at the simple molecule level only at cryogenic temperature (11), but, recently, single-molecule studies in ambient temperature environments are becoming possible (for a review, see ref. 12). This is crucial for studying biomolecules.

A rich example of the power of the fluorescence approach is provided by the study of the conformational and chemical dynamics of single enzyme molecules (13). Cholesterol oxidase is a 53-kDa enzyme that catalyzes oxidation of cholesterol by oxygen. The enzyme's active site, flavin adenine dinucleotide, is fluorescent in its oxidized form but not in its reduced form (Fig. 2*A*). The emission exhibits on–off behavior, each on–off cycle reflecting an enzymatic turnover (Fig. 2*B*). Therefore, enzymatic turnovers of single enzyme molecules can be monitored in real time. Statistical analyses of the turnover trajectories make it possible to see whether directly chemical dynamics at the single-molecule level follows the mechanism inferred by bulk studies on populations of molecules. Crudely speaking, it does! For the first time, one sees that enzymic catalysis follows the sequence of events inferred by Michaelis and Menten 10 ago. But there is important fine structure to the statistics not in the Michaelis–Menten Mechanism!

The emission on-times (or off-times) for the reduction (or oxidation) of flavin adenine dinucleotide are, as expected, stochastic in nature. If the oxidation of flavin adenine dinucleotide is a Poissonian process, the distribution of on-times is expected to be an exponential decay (the fitted line in Fig. 2*C*). Although the Michaelis and Menten mechanism would assume a single rate, Lu *et al.* observed a broad distribution of rates for many individual enzyme molecules (13). Similar static disorder of single-molecule enzymatic reaction rates has been reported previously (14, 15). Furthermore, the disorder turns out to be dynamic: i.e., the reaction rates of single molecules fluctuate as the molecule in its environment explores its energy landscape. In classical chemical kinetics, which follows Poisson statistics, when an enzyme molecule undergoes a turnover, it carries no memory of any previous turnovers and proceeds with a constant rate. One way to analyze data is to use a scrambled histogram as in Fig. 2*C*. This method of analysis is not particularly sensitive to memory effects. The disorder is difficult to pin down precisely. A more sensitive test can be made with the single-molecule turnover trajectory by using the autocorrelation function of the on-times (Fig. 2*D*), $r(m) = \langle \Delta\tau(0)\ \Delta\tau\ (m)\rangle / \langle \Delta\tau^2 \rangle$, where m is an index number for the turnovers in a trajectory and $\Delta\tau(m) = \tau(m) - \langle \tau \rangle$, and where the bracket denotes the average along the trajectory. For a Poissonian process, $r(m)$ is expected to be only a spike at time zero. The decay of $r(m)$ indicates that the reaction rate is slowly fluctuating, which can be accounted for by slow conformational fluctuations (13, 16). This intermittency phenomenon, subtle as it is, is hidden in the ensemble-averaged measurements on bulk samples at room temperature (16). This gives an example of the new questions that must be asked (and can be answered) by using the single-molecule approach.

What other specific phenomena may be hidden in the averaging necessitated by ensemble averaging in most kinetic studies? We are only at the beginning, but it is clear there is much to be discovered of a fundamental nature about complex molecules viewed as individuals. Perhaps equally important will be the idea of single molecule control. Now that experiments interact with molecules at an individual level, we can try to control them as individuals, not as populations. A molecule under active control by an adaptive environment will be a new beast. Such tamed molecules may well resemble much more the elegant engineered machinery of everyday experience than the unruly, wild molecules we are used to studying, today.

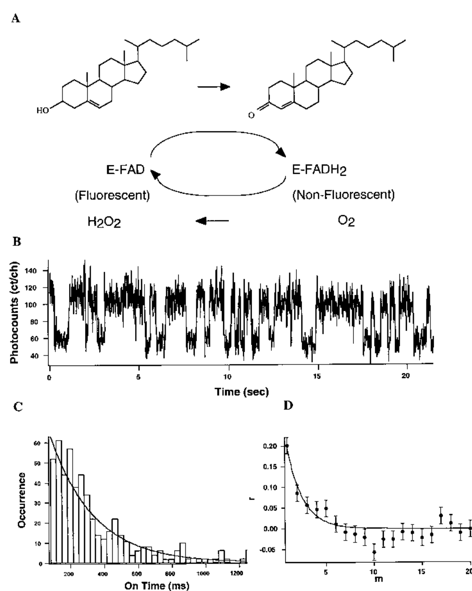

FIG. 2. (*A*) Enzymatic cycle of cholesterol oxidase, catalyzing oxidation of cholesterol by oxygen. (*B*) Real-time observation of enzymatic turnovers of a single cholesterol oxidase molecule. (*C*) Histogram of on-times from the turnover trajectory of a single cholesterol oxidase molecule. (*D*) Autocorrelation function of on-times derived from a trajectory, indicating a slowly fluctuating rate.

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