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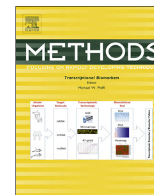

Practical single molecule force spectroscopy: How to determine fundamental thermodynamic parameters of intermolecular bonds with an atomic force microscope

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ABSTRACT

Single molecule force spectroscopy involves loading a chemical bond using an atomic force microscope and measuring the rupture forces required to break that bond. In 20 years since its inception this technique developed into a robust way to extract a nearly complete set of the information about the bond that includes the bond energy, the kinetic parameters of the bond, and the geometry of the transition state. In this article we review the basic physics of the measurements, the model that is used for data interpretation, and go over the ways to extract the bond information from the experimental data. We also discuss several practical aspects of the measurements that are helpful to the planning and analysis of single molecule force spectroscopy experiments.

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1. Introduction: 20 years of force spectroscopy

The current paradigm for understanding molecular level events in nature is firmly rooted in the laws and concepts established in Gibbs' seminal works that founded modern chemical thermodynamics. An astonishing variety of intermolecular bonding between chemical species creates chemical driving forces that power most of condensed matter processes. Early on, our ability to characterize these interactions in a meaningful quantitative way had to rely on bulk-scale calorimetric, or spectroscopic techniques. The advent of nanoscience and development of single molecule methods in the past 20 years brought to the table a whole new set of tools that opened up new avenues for exploring and manipulating molecular interactions on an unprecedentedly detailed scale [1–4]. These approaches gave researchers the ability to probe and map localized interactions [5], quantify extremely high and low binding affinities [6], and explore the potential energy surfaces of chemical and biological interactions [7] using methods that eliminate ensemble averaging inherent to the bulk techniques.

For the past 20 years since the pioneering works by Gaub and Lieber groups [5,6] chemists and biophysicists have been entertained by a deceptively simple idea of using nanoscience tools for direct characterization of an intermolecular bond simply by grabbing both sides of that bond, pulling it apart, and measuring the force or, ideally, the work that is required to accomplish this process.

The invention of scanning probe microscopy introduced two critical technical advances that made these experiments a reality: piezoelectric scanners of an atomic force microscope (AFM), that can position and move a sample in space with angstrom-level precision, high stability, and a wide range of speeds; and microfabricated AFM cantilevers [8] with stiffness low enough for measuring molecular interactions. By now AFM became a ubiquitous instrument in today's nanotechnology laboratories, and it is not necessary to describe it in detail; suffice to say that it uses a sharp tip mounted on the end of a microfabricated flexible cantilever to probe the sample mounted on a piezoelectric scanner that controls the sample position in all three spatial dimensions (Fig. 1A). Typical potential energy gradients of intermolecular interactions range from 10^{-12} N to 10^{-7} N [9], and typical AFM cantilever deflection values range between 0.1 and 100 nm. Thus, to be useful for force spectroscopy measurements, AFM cantilevers need to have spring constants in the range of 0.01–1 N/m, and many commercial AFM probes deliver stiffness in this range.

Early on the scanning probe microscopy community recognized the potential of the AFM for measuring bond strengths [10–12], used it to probe a number of interactions between well-defined chemical functionalities [13], protein–protein pairs [6], and colloidal particles [14], and quickly built up an extensive database of the measurements. Since that time, the measurement precision and scope have greatly expanded [4], and the technique has explored interactions that range from binding to live cell surface receptors

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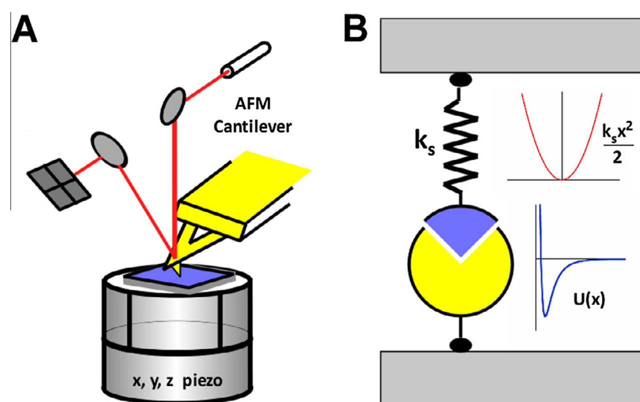

Fig. 1. (A) Schematic of an atomic force microscope. (B) The equivalent mechanical diagram of the single molecule force spectroscopy experiment showing the parabolic potential of the cantilever spring, and the potential energy surface of a bond that is being loaded.

[15] to forces between single functional groups [16]. An important realization, which shaped the practice of the force spectroscopy experiments in a profound way, was that soft levers that limit the damage to the sample and the soft connecting linkers that help to isolate the relevant interactions preclude the possibility of tracing the interaction potential with the probe. Consequently, most of the force spectroscopy measurements are restricted to measurement of the rupture forces and the key questions that faces the researchers is: *How to connect the rupture force values to fundamental parameters of the bond such as: equilibrium energy of the bond, its lifetime, and potential energy surface geometry of the bond?*

These question have remained relevant almost since the technique's inception; for example, almost two decades ago two different laboratories reported rupture force values for the interactions of tips and samples functionalized with methyl-terminated alkanethiols in pure ethanol – a seemingly simple and robust system – that differed by a factor of 2.5 (and both laboratories expressed high confidence in the measured values) [5,17]. The reported discrepancies in biological interaction values could be even higher, so it was clear that proper analysis should find a way around this problem.

This article is not an overview of the recent experimental achievements in force spectroscopy; they are addressed in many reviews and books [18–21], and the field has been too big for too long to fit into a space of a single article. It is also not an overview of the theory of single molecule force spectroscopy. Instead we intend to present a concise overview of the analysis that aims to extract the fundamental thermodynamic and kinetic parameters of a single bond from the force spectroscopy data. We start with a qualitative overview of the dynamics of breaking a single bond under external load, and then we present a detailed model that predicts rupture forces that a researcher can expect to measure. We then discuss some of the practical aspects of doing these measurements, and finish by describing some of the general considerations of statistics and noise relevant to the data analysis.

2. Pulling a single bond: a qualitative description of a single molecule force spectroscopy measurement

In a simplified physicist's view a force measurement experiment is equivalent to stretching two or more elastic elements connected in series (Fig. 1B). One of these elements corresponds to a bond between the interacting molecules (or more strictly, a projection of the bond potential to a pulling coordinate), and another to a cantilever spring or to a combination of the AFM cantilever spring and a nonlinear stiffness of a polymer linker that attaches the interacting species to the probe and sample surfaces. In virtually all measurements a very rigid piezoelectric scanner stretches this construct until the bond ruptures and the instrument records the value of the applied force at which this rupture occurs. In the simplest case, we can represent the tip–sample interaction with a single-well potential, and assume a parabolic potential of a Hookean spring for the cantilever (Fig. 1B). The equilibrium position of the cantilever is then determined by a sum of these two potentials, and as the piezo scanner moves the cantilever away from the surface, a secondary minimum emerges on the potential energy surface (Fig. 2). This transition to a secondary minimum is what enables a sudden jump of the cantilever away from the surface during retraction. Therefore, to understand the measurement, we need to consider the kinetics of the transition from the bound state to the unbound state (the secondary minimum). As the loading force changes much slower than the frequency of the thermal fluctuations, the unbinding transition is driven mainly by thermal fluctuations and the role of the external force is limited to changing the overall potential energy landscape of the system. In other words, force-induced bond rupture in the atomic force microscope is simply a *transition from the bound state into an unbound state over a potential energy surface that is constantly modified by the time-dependent potential of the loading spring.* The moment of this transition defines the measured rupture strength of the bond. This fundamentally kinetic view of the unbinding process, that was first articulated in the pioneering works by G. Bell and E. Evans [22,23], is the first key concept that is required to understand the physics of force spectroscopy experiments.

The second key concept is that the loading potential does not create the secondary minimum immediately; instead, at the initial phases of the loading process, it forms a "wall" on the potential energy surface that prevents the bond from leaving the bound state (Fig. 2A and B). Although thermal fluctuations will always break an unperturbed biological bond under no load after an extended period of time (a process that defines the bond's natural lifetime), in a force spectroscopy experiment the probe potential will prevent rupture. Only after the probe potential moves farther away from the bond minimum (Fig. 2C and D) does the secondary minimum form and the bond can rupture. This process leads to the

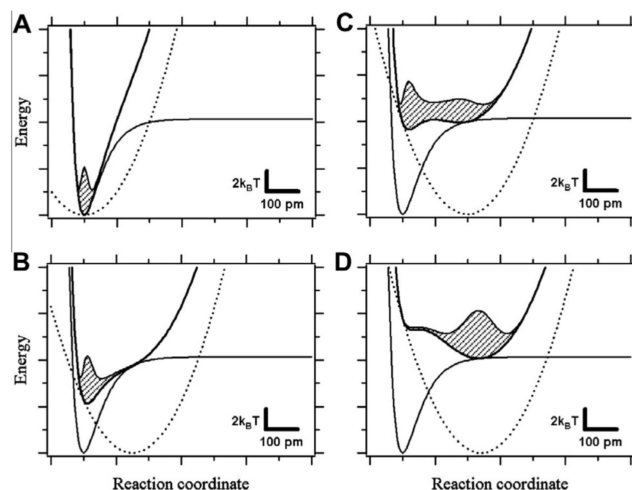

Fig. 2. Potential energy surface (thick solid line) and the density of states (shading) at different stages of single molecule force spectroscopy experiment. The bond potential is indicated by a thin solid line, the loading spring potential by a dotted line. The secondary minimum on the potential energy surface emerges only when the loading spring moves far enough from the bond potential (panel C).

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