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Accelerated Articles

Dissociation of Ligand−Receptor Complexes Using Magnetic Tweezers

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We present a new tool for measuring ligand−receptor complex bonds at the single-molecule level using magnetic tweezers. Our apparatus allows massively parallel (100−1000) measurements on many single complexes perturbed by constant forces. Compared to other single-molecule techniques, our method is simple, inexpensive, robust, and widely compatible with other techniques. We immobilized specific receptor molecules on the surface of superparamagnetic beads and corresponding ligand molecules on a fixed surface. The beads were allowed to contact the surface so that ligand−receptor binding occurred. A permanent magnet then generated a constant force that pulled the receptors away from the ligands. The rates at which bound species separated at various forces allowed us to characterize the potential energy landscape of the bond and extrapolate bulk solution kinetic rates and transition-state distances. These values agreed with those obtained using bulk and single-molecule methods.

This paper describes the design of a new tool for a parallel measurement of ligand−receptor dissociation. By applying a constant force, we can tilt the energy landscape therefore increasing the rate of dissociation as the force is increased. We immobilized the proteins behaving as receptors on superparamagnetic beads and allowed them to contact the ligand molecules adsorbed on a flat surface. We measured the number of beads coming off this surface at several forces versus time and calculated the rates of dissociation from-single exponential decay curves. Finally it was possible to calculate the dissociation rate constant at zero force for several complexes.

The study of ligand−receptor interactions is relevant to many fields including cellular biology, drug design, and diagnostics.[1−3] These specific interactions are composed of multiple short-range noncovalent bonds between ligands (often small molecules) and geometrically complementary recognition sites on receptors (usually proteins). These bonds may have electrostatic, van der Waals, dipole−dipole, and entropic contributions and can involve conformational changes. Specific interactions can be characterized by kinetic rates and affinities, thermodynamic free energies, and potential energy landscapes along the association and dissociation pathways. Ensemble-averaged information about specific bonds can be obtained using surface plasmon resonance (SPR), calorimetry, radioactive or fluorescence labeling, and acoustic wave biosensors. In recent years, significant progress has been made to manipulate and detect single molecules.[4−6] These measurements give information about the energy landscape and the trajectory of the dissociation path at the atomic scale, shedding light on the dynamic behavior of molecules lost in traditional ensemble measurements. Moreover, single-molecule methods provide information on movements, interactions, configuration changes, and other dynamical properties involved in single-molecule processes and subpopulations that would be otherwise hidden in ensemble techniques.

New insights into single-molecule behaviors have been provided by experiments where a force is applied to a molecule or complex. Such measurements have given us insight into the forces involved in biochemical reactions in vivo. Intuitively, when the force is used to pull two interacting molecules apart, the lifetime of the bond is drastically reduced. This happens because the bond is composed of many weak noncovalent interactions with associated activation energy barriers, and the applied force changes the heights of these barriers. Conceptually, the bond energy landscape is tilted by the applied force, allowing thermal noise to overcome

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activation energies and break the overall bond. For most experiments in which single-molecule bonds are ruptured, different force loading rates are applied to give a dynamic spectrum of the bond strength. This dynamic force spectroscopy (DFS) has been used to probe the potential energy landscape of complex macromolecular bonds for single molecules.[1] This approach investigates far from equilibrium conditions. In DFS, a probe and a substrate are brought into contact so that a specific bond is formed. The probe is then removed at a constant loading rate, and this rate is varied over several orders of magnitude to determine the locations of kinetic barriers (transition states) of ligand−receptor complexes. The three main techniques used in DFS measurements are (i) atomic force microscopy (AFM),[7−9] (ii) biomembrane force probes (BFP),[10,11] and (iii) optical tweezers.[12−14] AFM, though originally developed to image surfaces, has been widely used to evaluate single-molecule interactions such as ligand−receptor, protein−protein, and nucleic acid complementary strand interactions, as well as structural transitions in polysaccharides, DNA, and multidomain proteins.[7−9] BFP experiments have similarly been used to study energy landscapes of ligand−receptor bonds.

Alternative methods such as magnetic tweezers[15−19] and single-molecule fluorescence microscopy[20,21] have demonstrated that single-molecule events can be followed in real time and in vivo. Many of these single-molecule force studies have followed one interaction at a time, making it difficult or time-consuming to obtain good statistics on the behavior of rare stochastic events. Measurements made in parallel can shorten acquisition times and reveal events and states that occur infrequently.[17,18] We present here a new tool that allows many (100−1000) single ligand−receptor complex measurements in parallel, using magnetic tweezers capable of applying a wide range of biologically relevant forces (0.1−160 pN). Our method is well suited to probing specific ligand−receptor bonds crucial for molecular recognition and excels at applying forces to biopolymers such as single- and double-stranded DNA (dsDNA), RNA secondary structures, and actin filaments. Magnetic tweezers are able to provide information about unbinding events that occur at or near equilibrium. These events are thermodynamically reversible and represent elastic deformations. For small constant forces, the dissociation reaction takes place along the minimum energy path, as all energy states are available. Unlike optical tweezers, AFM, and BFP, our parallel magnetic tweezers can exert forces using a simple, inexpensive, and robust apparatus that requires minimal alignment and is compatible with microfluidics.

We have used parallel magnetic tweezers to unzip dsDNA at a range of constant forces and temperatures.[17,22] In agreement with theoretical predictions made by Lubensky and Nelson,[23,24] the separation of dsDNA with a random base sequence did not proceed at a constant rate, but opened in a sequence of sharp jumps separated by large plateaus of little or no activity. This is because strand separation requires dissociation of many base−base interactions of varying strengths, so the overall unzipping will pause at a sequence of potential energy minimums until thermal activation carries the separation past transition-state energy barriers. In this paper, we demonstrate that parallel magnetic tweezers can be used in a different geometry to count bound/unbound states for many more single-molecule interactions simultaneously, providing different information about the potential energy landscape of noncovalent bonds. We applied our method to the study of the prototypical ligand−receptor pair (strept)-avidin−biotin, by functionalizing surfaces and superparamagnetic beads. The bound complexes were separated by constant forces, allowing us to extrapolate kinetic rates and distances to transition states in the absence of force. These values were compared with those obtained using other methods.

EXPERIMENTAL SECTION

Reagents. The 4.5-μm superparamagnetic beads (Dynabeads M-450 tosyl activated) were functionalized with (strept)avidin following the suggested protocol (Dynal). This derivatization is based on covalent attachment of amine-containing lysine residues of the protein to beads bearing reactive tosyl groups. Briefly, an aliquot (0.4 mL) of the bead suspension was placed in a magnetic rack and washed three times with 0.1 M phosphate buffer pH 8.4 and then resuspended in 1 mg/mL streptavidin or avidin and incubated overnight at 37 °C with continuous mixing. The beads were washed twice with phosphate-buffered saline (PBS; 10 mM phosphate and 150 mM NaCl) containing 0.1% (w/v) bovine serum albumin (BSA) and incubated with 0.5 mL of 0.2 M Tris buffer pH 8.5 and 0.1% (w/v) BSA at 37 °C. Finally, the beads were washed twice in PBS/BSA and stored in 0.4 mL of PBS/BSA buffer at 4 °C. The beads were thoroughly washed before each experiment and diluted 1:10 with PBS.

The ligands used were biotinylated bovine albumin (b-BSA, Sigma) or iminobiotinylated bovine albumin (iminoBSA) prepared by covalent reaction between 2-iminobiotin and *N*-hydroxysuccinimide ester (NHS-iminobiotin) (Sigma) and BSA (Sigma): 1 mg of BSA was dissolved in 0.5 mL of 50 mM sodium borate buffer pH 8.2 and 20 μL of 0.25 mg of NHS-iminobiotin dissolved in dimethyl sulfoxide was added. The mixture was vortexed for

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several minutes and kept for 2 h at 4 °C. Finally, the modified protein was purified by ultrafiltration using Microcon YM-10 filters (MW cutoff 10 000). The protein b-BSA or iminoBSA was diluted with BSA (1:150) prior to overnight adsorption on 22-mm^2 plastic slides (VWR).

Experiment. A 10-μL aliquot of the bead suspension was placed on a plastic slide, and the beads were allowed to settle for 30 s. A constant force of 10 pN was applied for 2 min in order to remove excess and nonspecifically attached beads. A higher force was then applied, and the number of bond-breaking events was measured in 1-s intervals for 10 min. All the measurements were performed in PBS buffer pH 7.4 containing 0.05% Tween 20.

Apparatus. In our instrument, the magnetic field gradient is produced by a stack of four neodymium magnets measuring 6.4 × 6.4 × 2.5 mm each. The total magnetic field is approximately that of a solenoid with its long axis in the z direction. The field along the z axis is purely in the z direction and uniform relative to the solenoid axis to within a few percent. The beads are superparamagnetic and thus do not retain any residual magnetization or attract each other in the absence of an external magnetic field. When an external field is applied, the beads experience a magnetic force $m\cdot\text{grad}\ B$, where B is the magnetic field and m is the magnetic moment on the bead.[18] This force on a bead in our apparatus is exclusively in the z direction and varies by less than 1% over the sample region monitored in the experiment.[18] The magnitude of the applied force on the beads is controlled by the distance between the magnet and the surface. This experimental setup allows us to follow hundreds of beads in parallel.

Bead tracking was performed with an inverted microscope (objective lens 10×, 0.25 NA) focused on the ligand-coated surface. As ligand−receptor bonds dissociated, the beads were carried out of the focal plane of the microscope. Images were acquired by a camera, captured by a video frame grabber, and analyzed using custom image software written in IDL (Research Systems Inc.). By counting the decrease in the number of beads across successive image frames, we obtained the unbinding rate for a given applied force.

Calibration. The relationship between magnet distance and applied force was determined using Stokes drag on a magnetic bead. The beads were washed in water and dried slowly at 37 °C, then resuspended in glycerol, and placed in a square capillary (i.d. 1 mm). The velocity of a bead at different distances from the magnet was measured, and the velocities of several (5−10) beads were averaged to obtain a mean force. The values quoted for the force are the average values for a given magnet distance.

RESULTS AND DISCUSSION

Figure 1a shows a schematic of the magnetic tweezers apparatus. The magnet exerts a force on the superparamagnetic beads functionalized with the receptor protein (strept)avidin, whereas the ligand was adsorbed to the surface in contact with the beads. In each experiment, a perpendicular force is applied to hundreds of beads. Thus, the uniform magnetic field acting on each individual bead creates an array of tweezers for the manipulation of individual biomolecules. The magnets were held in a vertical position with respect to the microcell, and a three-axis translation stage was used to center the magnet so that forces were only exerted perpendicular to the surface. To measure the unbinding rate, the microscope was focused on the bottom surface

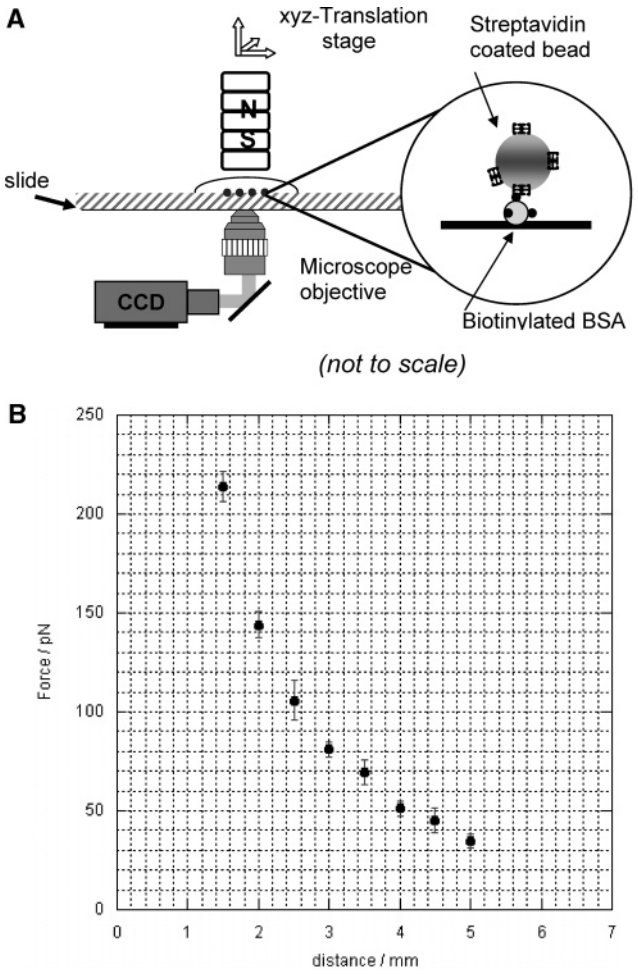

Figure 1. (A) Schematic diagram of the apparatus. The beads are allowed to contact the substrate surface while being observed through an inverted microscope objective. The magnet can be moved separately over the sample. (B) Force applied to the 4.5-μm beads suspended in glycerol and calculated from the Stokes drag. Points are averages of 5−10 beads for each magnet−surface distance. The error bars for the distance values are less than the size of the symbols.

of the microcell and the number of beads remaining was counted as a function of time.

The range of forces that can be achieved with our current instrument depends on both the magnets and the magnetic moment of the beads. In this work, we used 4.5-μm superparamagnetic beads and a stack of four permanent magnets. A calibration plot is shown in Figure 1b where the relationship between force on the beads and distance from the magnet was determined. This measurement can be done by placing a suspension of beads in glycerol in a glass capillary and following the movement of the beads toward the magnet that is approached laterally. Thus, for each position measured with micrometer precision of 10 μm and an uncertainty of ±100 μm, an average of the velocity of 5−10 beads was obtained and expressed as a force using the Stokes drag equation. As can be seen from the plot, the variation in the force was ∼±10% in contrast with the larger variation observed with smaller beads used in other experiments.[17] This spread in the force is mainly determined by the variation in iron content and to a lesser extent by the differences in size between the beads (less than 5%) as well as the variation of the force in the $x-y$ direction (∼1%).

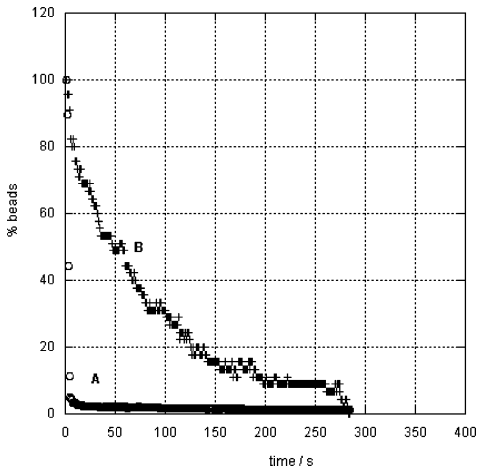

Figure 2. (A) Decrease of the number of beads on the surface with time for a negative control: streptavidin-coated beads on BSA-coated substrate at 5 pN; (B) dissociation of streptavidin-coated beads bound to biotinylated BSA at 45 pN.

Three controls were used to confirm that dissociation rates were being measured for specific single ligand−receptor bonds and not other surface interactions. One negative control consisted of a BSA-coated surface containing no ligands. In the other two controls, the receptors or ligands were blocked by saturation with free biotin or (strept)avidin, respectively, before beginning the experiment. In all these controls, the beads were completely removed by low forces (5−10 pN), with typical backgrounds of 2−4% nonspecifically bound beads after 2 min.

Figure 2 shows a typical plot of the number of streptavidin-coated beads versus time for a plastic slide coated with BSA (negative control) or biotinylated BSA. Beads bound to the BSA surface nonspecifically were removed rapidly at low forces (5 pN), whereas the dissociation of streptavidin−biotin bonds exhibited a longer time constant even at higher forces (45 pN). This ligand−receptor dissociation curve is fit by an exponential to obtain the dissociation rate for that applied force.

In dynamic force spectroscopy, a variable force is applied to the bound species. This approach investigates far from equilibrium conditions. By varying the loading rate across several orders of magnitude, both the dissociation rate of the reaction and the number of inner reaction barriers can be calculated. Alternatively, magnetic tweezers apply a constant force; therefore, information about the bond is revealed by tracking the number of unbinding events as a function of time, which can be done by analyzing images of beads by computer. If the focal frame of the imaging system includes the bottom surface of a glass cell, beads connected to bonds that break will be pulled out of the focal frame; thus, the problem of measuring the unbinding rate is reduced to counting the decrease in the number of beads across successive frames of a movie.

According to the law of mass action, the reversible reaction L + R ↔ LR can be described as two reactions that occur at characteristic rates that depend on reactant concentration:

$$\mathrm{d[LR]}/\mathrm{d}t = k_{\mathrm{on}}[\mathrm{L}][\mathrm{R}] \tag{1}$$

$$\mathrm{d[L]}/\mathrm{d}t = \mathrm{d[R]}/\mathrm{d}t = k_{\mathrm{off}}[\mathrm{LR}] \tag{2}$$

where k_{on} and k_{off} are constant functions of temperature. The

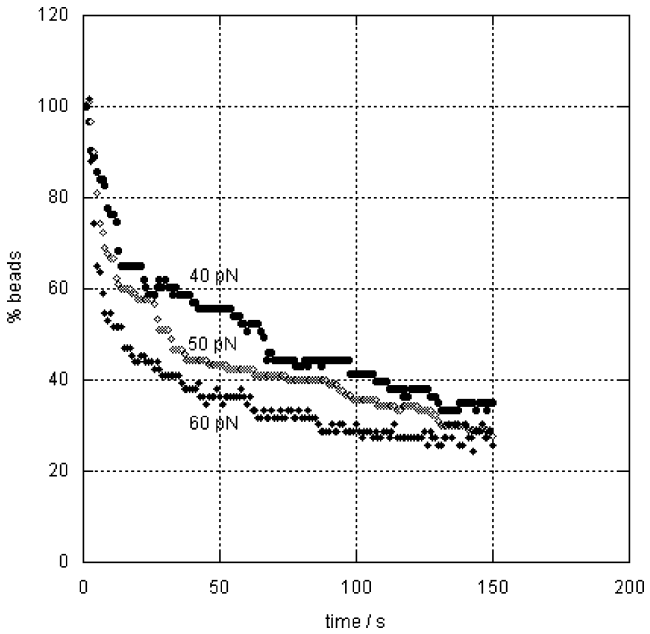

Figure 3. Decrease of the percentage of avidin-coated beads on the biotinylated-BSA modified surface with time at different forces: (●) 40, (◇) 50, and (◆) 60 pN.

change in free energy to the transition state (the height of the reaction barrier) can be related to the kinetic rates by

$$k_{\mathrm{on}} = v_{\mathrm{on}} \exp(-\Delta G^{\ddagger}_{\mathrm{on}}/k_{\mathrm{b}}T) \tag{3}$$

$$k_{\mathrm{off}} = v_{\mathrm{off}} \exp(-\Delta G^{\ddagger}_{\mathrm{off}}/k_{\mathrm{b}}T) \tag{4}$$

where k_{on} and k_{off} are typically measured in bulk solution and v_{on} and v_{off} depend only on the molecular details of the interaction.

Magnetic tweezers are able to provide information on k_{off}, as the magnetic force shifts equilibrium toward unbound states while preventing further binding. According to Bell's model,[25] the free energy of the transition state is decreased by the magnetic work done on the bond:

$$W = -F_{\mathrm{magnetic}}x \tag{5}$$

$$\Delta G^{\ddagger}\,(F) = \Delta G^{\ddagger}(0) - Fx \tag{6}$$

where x is the reaction coordinate which (in a simple interaction) is the distance that A and B must be separated to pass a kinetic barrier and break the bond. This implies that the off rate changes exponentially according to the applied force:

$$k_{\mathrm{off}}(F) = k_{\mathrm{off}}(0)\,\exp(Fx/k_{\mathrm{b}}T) \tag{7}$$

Figure 3 illustrates the effect of force on the dissociation rate. After applying a low force to eliminate nonspecifically bound beads, the force was increased and the dissociation of ligand−receptor bonds was measured. The dissociation rate increases as the force is increased. The off rate, k_{off} (F) was determined by

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counting the number of unbroken bonds as a function of time and fitting these data to a monoexponential:

$$N(t) = N(0)\ \exp(-tk_{off}(F)) \quad (8)$$

For forces of <60 pN, the decrease in the number of bound particles as a function of time could be fit well using a single-monoexponential function. However, at forces above 60−70 pN, a better fit was obtained using biexponential decay curves that were the sum of two monoexponentials with different characteristic decay times. Such multiple decay times will result if the potential for a single bond has more than one minimum or if each bead is bound to the surface by more than one ligand/receptor pair. Even though the ligand is significantly diluted with albumin, some beads may still be bound by multiple ligand/receptor pairs, a problem that is exacerbated by the multiple biotins that are covalently attached to each biotinylated BSA. We tried to reduce the number of multiple bonds by incubating the beads in different concentrations of free biotin so that several streptavidin (avidin) sites were occupied by the ligand before starting the experiment. The rate constants obtained from the biexponential curves at high forces did not vary upon changing the biotin concentration between 0.05 and 10 nM whereas the fraction of beads that decayed at the longer decay decreased as the concentration of free ligand increased, suggesting that the longer decay may be attributed to multiple bonds, rather than multiple potential minimums for a single bond. The insensitivity of both rate constants to free ligand concentration suggests that it is possible to obtain the single bond dissociation rates from the first exponential term in the higher force curves where multiple bonds may be playing a role.

Alternative strategies exist to ensure measurement of single bonds. The dilution of ligand molecules on surfaces can be easily controlled using self-assembling monolayers, where the mole fraction of the ligand is determined by the mole fraction in the solutions used to prepare the mixed SAM.[26] It has been shown that it is reasonable to assume that the composition of the SAM will be similar to the composition of the solutions, thus allowing dilution of the ligand on the surface so that multiple bonds can be avoided.

By varying the force used to disrupt the ligand−receptor bonds, it was possible to extrapolate k_{off} to zero force, corresponding to k_{off} in solution. Figure 4 shows the dependence of the dissociation rate versus force for streptavidin and avidin beads on biotinylated and iminobiotinylated BSA-coated surfaces. We calculated the unbinding reaction distance (x) for the three ligand−receptor pairs using Bell's model:

$$\ln k_{off}(F) = \ln k_{off}(0) + Fx/k_bT \quad (9)$$

We obtained k_{off} values of 9×10^{-5} and 1.4×10^{-4} s^{-1} and x values of 0.36 and 0.38 nm for avidin−biotin and streptavidin−biotin, respectively.

In these experiments, we were able to follow the time required for the dissociation of individual ligand−receptor complexes. Since the dissociation is a stochastic event driven by thermal fluctuations, to extrapolate one or several k_{off} values, it is necessary to average the individual behavior of many single-molecule dissociations, thus characterizing one or more dissociation pathways for a given ligand−receptor pair.

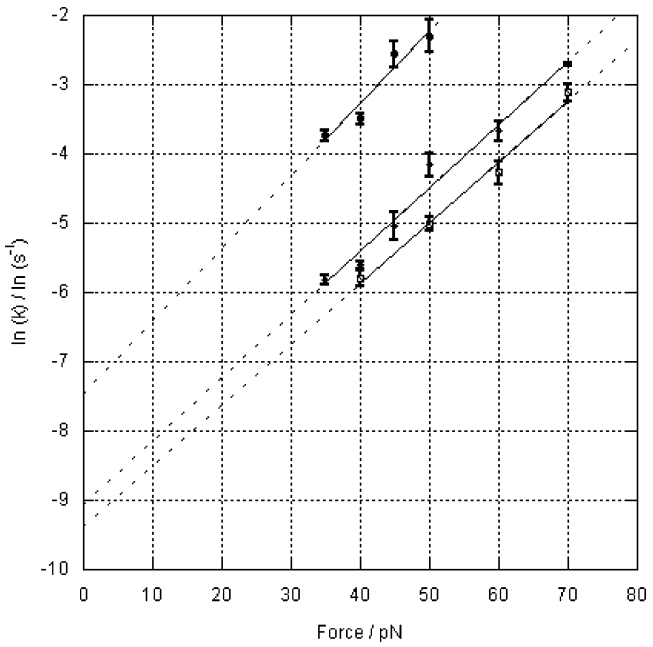

Figure 4. Dependence of the logarithmic values of the measured dissociation constants (k_{off}) on the applied force. The rate constants were obtained as explained in the text for beads coated with (□) avidin, (◆) stretptavidin on surfaces modified with biotinylated BSA, and (●) avidin-coated beads on surfaces modified with iminobiotinylated BSA.

The results can be compared with previously reported values obtained with other methods. Measurements using SPR have reported apparent dissociation constants for biotin−streptavidin between 1.37 10^{-4} and 3.3×10^{-4} s^{-1}. [27] The dissociation rate was larger than the solution value (3.3×10^{-6} s^{-1}) and has been attributed to unfavorable steric interactions between the adsorbed protein and the surface or entropic constraints imposed by the surface which determine lower energy barriers.[27] Monolayers with long spacers between the biotin molecule and the surface have been shown to decrease the dissociation rate, thereby approaching bulk solution values.[28] Several values have been reported in AFM experiments for the extrapolated dissociation rate for streptavidin (1.67×10^{-5} s^{-1}) and avidin (6.45×10^{-6}−10^{-3} s^{-1}).[29,30] Experiments using a biomembrane force probe give an avidin−biotin value of ~10^{-4} s^{-1} by extrapolating from the relationship between rupture force and loading rate.[31] Comparing x, the characteristic displacement of the ligand to achieve unbinding, is more difficult

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since this value depends on the loading rate. In the slow loading rate regime corresponding to our method, values of 0.49 and 0.53 nm have been reported for streptavidin−biotin and avidin−biotin, respectively.

Our method was further applied to a complex presenting a significantly different k_{off} using a biotin analogue, iminobiotin, which binds to avidin with lower affinity.[32] Figure 4 also shows the dissociation rate versus force for this ligand and avidin-coated beads. The kinetics and thermodynamics for iminobiotin−avidin binding have been studied with surface plasmon resonance, giving a k_{off} value of 3.1×10^{-4} s^{-1}.[32] Using our apparatus with avidin-coated beads and iminobiotinylated BSA adsorbed on a plastic substrate, we obtained a k_{off} value of 5.7×10^{-4} s^{-1} in reasonable agreement with the SPR method where a decrease in rate constants may occur due to mass transport limitations.[33]

In the present work, we have shown that polystyrene beads can be easily modified with different proteins following standard procedures whereas the ligand can be immobilized on a surface by covalent attachment to reactive groups on BSA. However, a more versatile strategy involves using self-assembled molecules allowing a better control for ligand concentration and orientation on the surface (to be published).

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CONCLUSIONS

We have developed a new tool for the measurement of single-molecule ligand−receptor dissociation events at constant forces. We followed hundreds of unbinding events in parallel, providing excellent single-molecule statistics in short times. The range of forces that can be applied depends on the beads and magnets. The number of bonds between each bead and the surface can be reduced to the single-bond level given a high dilution of ligand or a near-complete occupation of receptor sites using free ligand. The dissociation curves at low forces were fit to monoexponential functions whereas higher forces showed biexponential dissociation. The first rate constant obtained in the latter case did not vary upon changing the concentration of free ligand, suggesting it corresponds to the dissociation of single bonds. Our measured kinetic rates and transition-state distances are in good agreement with values reported using other methods.

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