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Label-Free Biosensing over a Wide Concentration Range with Photonic Force Microscopy

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We present a label-free biosensor that measures molecular interactions between biomolecules on the surface of a probe bead and substrate over a wide concentration range. This system is capable of detecting target biomolecules with concentrations varying from 10 nM to 0.1 pM, with high selectivity and sensitivity.

In recent years, several biomolecular detecting methods, based on the ligand–receptor binding reaction, have been introduced under various concentration conditions.[1] The motivation for performing the detection procedure with various biomolecule concentrations is that, in practice, many biomolecules in an organism are present in differing concentrations. The most commonly used method for low-concentration detection measurements is fluorescence labelling. An example of this is the scattered-light signal from a fluorophore attached to a biomolecular species, amplified by surface-enhanced Raman spectroscopy[2] or total internal reflection fluorescence microscopy[3] and collected by using confocal microscopy.[4] However, fluorescence labelling methods are quite laborious; additionally, optical damage, such as bleaching and quenching, can be a problem with this approach. For these reasons, label-free methods that use microcantilevers, microcavities, or nanoparticle probes have been suggested.[5]

Scanning force microscopy (SFM) has been proposed for label-free detection, because the target molecules can be detected by scanning functionalized probes over the surface of the target sample.[6] SFM provides higher selectivity than other methods, because it is capable of recognizing specific molecular interactions at the single-molecule level. Recently, Wei et al. successfully demonstrated label-free biosensing using atomic force microscopy (AFM), in which a functionalized tip attached to a cantilever can detect biomolecules by interacting with the surface and measuring the adhesion force between the biomolecules and substrate.[6a] In contrast, photonic force microscopy (PFM) uses a colloid bead trapped in optical tweezers.[7] Bartsch et al. successfully demonstrated biomolecular detection by using an optical trapping probe at the single-molecule level.[7d] Compared with AFM, PFM can readily be used to detect biomolecules, because the measurements are performed in liquid environments. Various types of probes can be used for the target sample. Probe replacement is performed simply by exchanging the solution.

Recently, we developed a PFM technique, in which both topographic and chemical mappings are simultaneously acquired through three-dimensional (3D) scanning. This system is capable of measuring the spatial distribution of target biomolecules on the surface and strength distribution of the molecular interactions at the single-molecule level.[7e] In this paper, by using the developed PFM technique, we demonstrate a label-free biosensing approach for biomolecule detection, with high sensitivity and selectivity over a wide range of concentrations, by scanning the probe bead vertically at thousands of different lateral positions.

To demonstrate biomolecular sensing, biotinylated DNA oligonucleotides and their complementary oligonucleotides were used as the probe and target molecules, respectively. The probe molecules were placed on the surface of a polystyrene bead (Figures 1a and S1), whereas the target molecules were placed on the bottom surface of a sample chamber (Figures 1a, S2, and S3). PFM was used to measure the molecular interactions between the probe molecules on the polystyrene bead and the target molecules on the surface of the sample chamber (Figures 1a, S3, and S4). Detailed methods for tracking the position of the probe bead and determining the contact position with the sample surface have been described previously in Ref. [7e]. By using the PFM technique, molecular interactions were detected at different lateral positions by the probe bead approaching and withdrawing from the surface. At each lateral position, as the probe bead approaches the surface, the probe and target molecules can bind to each other when the probe bead makes contact with the target surface. Once a probe and a target molecule bind, the probe bead does not detach from the target surface until molecular binding is ruptured by the optical trapping force, which occurs when the probe bead is withdrawn from the target surface.

Figure 1b shows a typical force-displacement (FD) curve for the probe bead when a target molecule is detected (additional FD curves are shown in Figure S5). The displacement and force are defined as the vertical position of the optical trap and the optical force acting on the probe bead, respectively (Figure S6). From the starting point A, the probe bead moves

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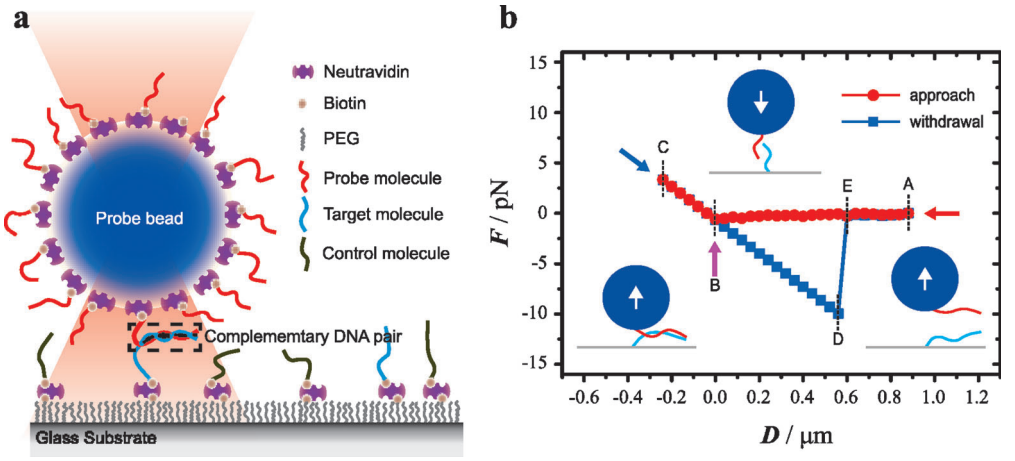

Figure 1. Representation of the sensing principle. a) Schematic diagram of the experimental design. A probe bead is manipulated three-dimensionally by using optical tweezers. When the probe bead approaches the substrate, the probe bead can bind to the substrate through hybridization of complementary pairs of oligonucleotides. The binding is broken when the probe bead retracts from the substrate. b) A typical FD curve; the probe bead approaches (blue) and withdraws (red) from the surface. The displacement is set to zero at the moment of contact between the bead and the surface.

toward the surface with a monotonous force near zero (from point A to point B). The contact position is recognized at position B, that is, when the bead reaches the surface. After contact with the surface, the force increases dramatically between positions B and C, because the probe bead has stopped on the surface while the optical trap continues to move down. After the movement direction of the optical trap is switched at position C, the force decreases, following the approach curve from C to B. The force decreases further after the optical trap passes position B (from point B to point D), because the probe bead still holds to the surface by DNA hybridization, whereas the optical trap moves upwards. As the optical force strengthens, it overrides the tension and breaks the DNA hybridization; the force returns along the approach curve (from D to E to A). This process represents one cycle of biomolecular detection at the surface.

To improve the selectivity of this biosensor, it is necessary to distinguish specific events from all of the detection events. The rupture force (F_R) in the FD curve quantifies the binding strength between the probe and target molecules. The distribution of F_R in Figure 2a is spread between 0 and 20 pN (11.9 ± 2.6 pN), where the target molecules are loaded. In contrast, the distribution in Figure 2b is spread between 0 and 7.5 pN (4.2 ± 1.7 pN), where the target molecules are not loaded. According to the results of Figures 2a and b, we can regard the events for which F_R was smaller than 7.5 pN as nonspecific events and excluded them from the detection events. In Figure 2c, the upper map shows the spatial distribution of all detection events from a sample with 10 pM target molecules. The lower map shows the spatial distribution of detection events, excluding nonspecific events. Various molecular interactions have been widely studied at the single-molecule level by using single-molecule force spectroscopy, which includes the rupture patterns of the molecular interactions.[8] We expect that the selectivity of detection can be improved further through advanced analysis of the shape of the FD curves, based on the single-molecule studies.

To investigate the detection range and sensitivity, we attempted to detect the target molecules on the surface at various concentrations and we changed the incubation times of the target molecules. Figure 3a shows the spatial maps of the detection events for different target oligonucleotide concentrations over a wide area. The number of detection events de-

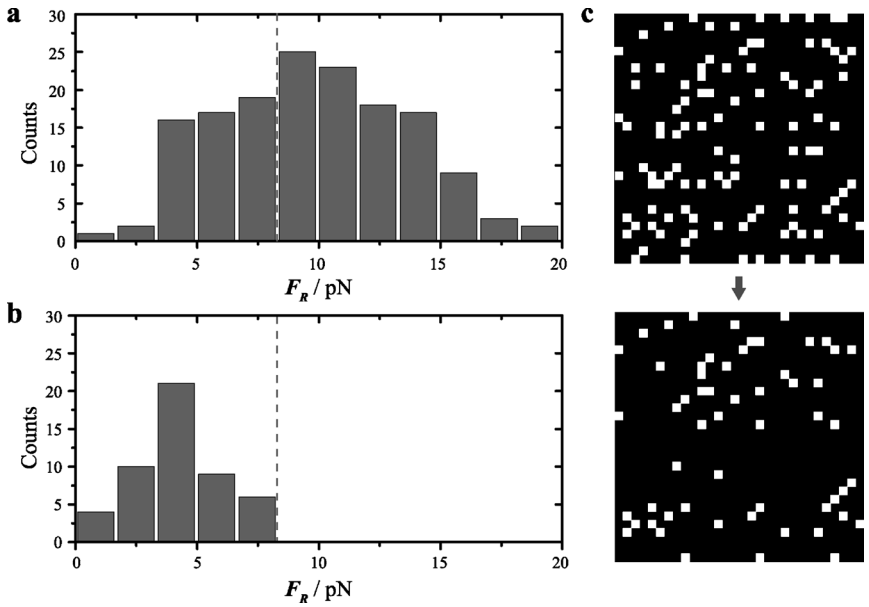

Figure 2. Distribution of molecular interactions. Distributions of F_R for the samples a) with complementary target molecules (10 pM Oligo-B) and b) without target molecules. c) Spatial maps of molecule detection (3 × 3 μm²). Yellow dots indicate positions where detection events are observed. The upper map shows all of the events; the lower map shows the events in which F_R is > 7.5 pN.

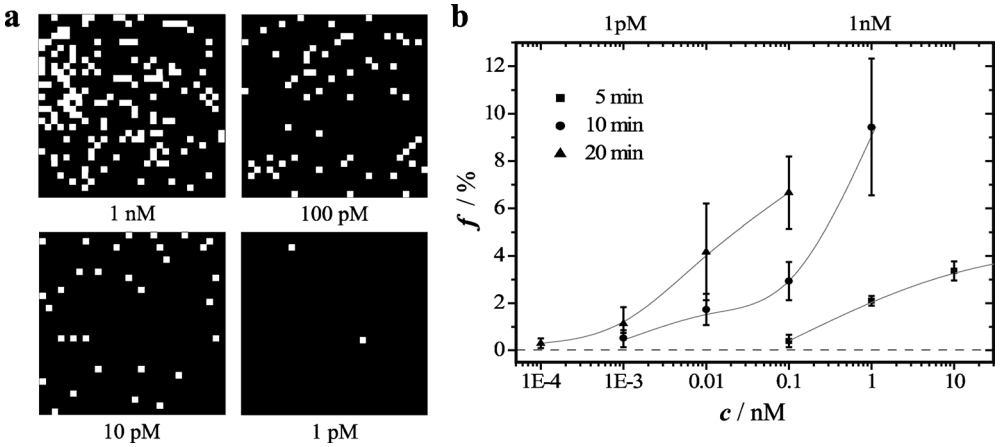

Figure 3. Detection range. a) Spatial maps (3 μm^2) of molecular detections at different concentrations of target molecules, where the incubation time of the target molecules was 5 min. b) Fraction of detection $f(c)$ at three incubation times of target molecules (5, 10, and 20 min). Solid lines are cubic B-spline lines for interpolation.

creased as the concentration of the target oligonucleotide decreased. We defined the fraction of detection $f(c)$ at target concentration c, which is the ratio of the number of detection events to the total number of vertical scans attempted. Figure 3b shows $f(c)$ at different incubation times of the target molecules (5, 10, and 20 min). Curves for $f(c)$ shifted toward lower concentrations as the incubation time increased, because more target molecules could attach to the surface as the incubation time increased.[9] This result indicates that target molecules can be detected in a range from 0.1 pM to 10 nM by choosing the appropriate incubation time for the target molecules. The lower limit of $f(c)$ approaches 0.1 nM, 1 pM, and 0.1 pM as the incubation time approaches 5, 10, and 20 min, respectively. To obtain these results, the number of vertical scans was set to 1000, to limit the total duration time of one target sample to 30 min.

Finally, we emphasize that our biosensing approach using PFM has several advantages. Firstly, variable probes can be used for multiplex detection when various target molecules are mixed in one sample, because we can easily replace the probes by exchanging the bead solution. Secondly, we can expand the observable strength range of molecular interactions, because the strength of the force exerted is controllable by changing the power of the trapping laser. Lastly, we can improve the detection limit by increasing the total number of vertical scans, because the target and probe molecules are not sensitive to time duration.

A limitation, however, is that fast and more frequent vertical scanning is required when the kinetics of the molecule interaction becomes weaker, because binding events become rare and the binding times become short. In terms of the instrument, the detectable strength of a molecule interaction is restricted by the upper limit of force of PFM, which is usually up to approximately 100 pN.

In conclusion, we developed a label-free biosensor that operates by using PFM, which has a wide detection range from 0.1 pM to 10 nM. We demonstrated that this biosensing approach has high selectivity and sensitivity through the detection of biomolecule interactions. The features assure accurate detection of small numbers of biomolecules. We anticipate that this biosensor will be used for the accurate and sensitive detection of molecular interactions over a wide concentration range.

Experimental Section

Probe and Target Molecules

Biotinylated DNA oligonucleotides and their complementary oligonucleotides were used as the probe and target molecules, respectively. The sequences of DNA oligonucleotides (Integrated DNA Technologies) were Oligo-A: 5′/ACT TAC CCA GTA AAA ACT TAC CCA GTA ATT TTT TTT TTT TTT TTT TTT TTT TTT TT/Biotin/−3′, Oligo-B: 5′/Biotin/TTT TTT TTT TAT TAC TGG TGA AGT TAC TGG TGA AGT/−3′, and Oligo-C: 5′/Biotin/TTT TTT TTT TTT TTT TTT TTT TTT/−3′. The 30 nucleotides at the 5′-end of Oligo-A were hybridized with the 30 nucleotides at the 3′-end of Oligo-B, for which the melting temperature (T_m) was 55.8 °C and the Gibbs energy, ΔG, was −22.24 kcal mol^{-1} at 50 mM Na^+ conditions. In this experiment, Oligo-A and Oligo-B created a zipping structure between the probe bead and target surface, because the 3′-end of Oligo-A could bind to the probe bead and the 5′-end of Oligo-B could bind to the target surface by biotin–avidin conjugations. The control DNA (Oligo-C), which does not interact with the probe DNA (Oligo-A), was used as a negative control.

Preparation of Probe Beads

Probe molecules were placed on the surface of a polystyrene bead (Figure S1). Firstly, 1 μm-diameter polystyrene beads, coated with streptavidins (STV), were dispensed into a 10 mM phosphate buffer saline (PBS) solution. To remove free STV, the bead solution was centrifuged at 10000×g (in which g is the relative centrifugal force) for 5 min and then the supernatant was removed. This process was repeated three times. The polystyrene beads were then incubated with biotinylated probe DNA Oligo-A in 10 mM *tris*-ethylenediaminetetraacetic acid (TE) solution for 30 min at 4 °C. To remove the unreacted Oligo-A, the solution was centrifuged for 5 min and the supernatant was discarded. This process was repeated three times.

Preparation of Target Samples

Target molecules were placed on the bottom surface of a sample chamber (Figures S2 and S3). The sample chamber was produced by sandwiching two glass slides with a thin spacer, which allowed easy exchange of the solution by pipetting. To suppress nonspecific binding between the glass substrate and the probe bead, polyethylene glycols (PEGs), with a small portion of biotinylated PEGs, were layered onto the glass surfaces by using a succinimidyl amide reaction, in which the glass slides (BK7) were pre-cleaned with potassium hydroxide (KOH) and silanized with aminosilane. The TE solution containing a high concentration of neutravidins (NTVs) was incubated for 5 min; the NTVs became bound with biotinylated

PEGs on the surface, resulting in the capture of additional target molecules. After washing the buoyant NTVs, the solution with biotinylated target molecules (Oligo-B) was injected and incubated for several minutes, depending on the concentration. Finally, a 100 nM Oligo-C solution was incubated for 5 min to block NTVs (which do not react with target molecules), which was then washed with the TE solution. The target molecule concentration was controlled by dilution with TE solution, which is commonly used as a buffer solution for DNA oligonucleotides.

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Keywords: biosensors · optical tweezers · photonic force microscopy · scanning force microscopy · single-molecule studies

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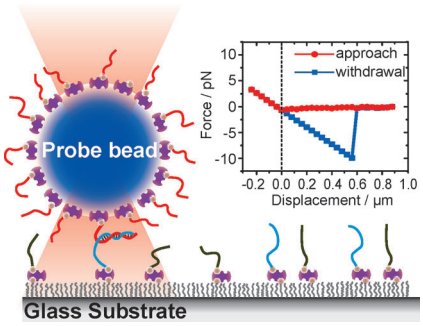
COMMUNICATIONS

Don't label me: A label-free biosensor that uses photonic force microscopy, which measures molecular interactions between biomolecules on the surface of a probe bead and substrate, is presented. The biosensor exhibits both high selectivity and high sensitivity.



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