Detection and localization of individual antibody–antigen recognition events by atomic force microscopy

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ABSTRACT A methodology has been developed for the study of molecular recognition at the level of single events and for the localization of sites on biosurfaces, in combining force microscopy with molecular recognition by specific ligands. For this goal, a sensor was designed by covalently linking an antibody (anti-human serum albumin, polyclonal) via a flexible spacer to the tip of a force microscope. This sensor permitted detection of single antibody–antigen recognition events by force signals of unique shape with an unbinding force of 244 ± 22 pN. Analysis revealed that observed unbinding forces originate from the dissociation of individual Fab fragments from a human serum albumin molecule. The two Fab fragments of the antibody were found to bind independently and with equal probability. The flexible linkage provided the antibody with a 6-nm dynamical reach for binding, rendering binding probability high, 0.5 for encounter times of 60 ms. This permitted fast and reliable detection of antigenic sites during lateral scans with a positional accuracy of 1.5 nm. It is indicated that this methodology has promise for characterizing rate constants and kinetics of molecular recognition complexes and for molecular mapping of biosurfaces such as membranes.

The invention of scanning force microscopy (SFM) (1) and its modification to optical detection of forces (2) has opened the exciting perspective of imaging the surface of living biological specimens (3–5). The additional potential of SFM for the study of molecular recognition, using a measuring tip with ligands bound, has recently gained much attention. The idea is to detect and study the binding of ligands on tips to surface-bound receptors by applying an increasing force to the complex that reduces its lifetime until it dissociates at a measurable unbinding force. So far, interaction forces were reported for the ligand–receptor pair biotin–avidin (6–8) and for complementary DNA nucleotides (9, 10). For these studies, SFM tips were covered with immobilized ligands. This strategy failed for antibody–antigen recognition (11), and the failure was attributed to the lack of molecular mobility and to unspecific tip–probe adhesion forces, obscuring specific interactions. Apart from detection and study of single recognition events, the concept of using SFM tips with ligands (“sensors”) has further perspectives: (i) for localizing individual recognition sites, (ii) for imaging their distribution at surfaces, and (iii) for combining recognition imaging by ligands with structural imaging by the tip, as a method for molecular mapping of the topography of biosurfaces. Advances toward this goal are expected from the realization of an appropriately designed sensor. The ideal sensor configuration appears to be a single ligand on a tip that is free to orient and move for unconditioned recognition during surface imaging by the tip. We approached this configuration by covalently coupling ligands to tips via a long flexible spacer molecule at a sufficiently low ligand concentration so that about one ligand is expected to have access to surface-bound receptors. In this report, we use an antibody as the sensor molecule and demonstrate the suitability of this sensor design for detection and characterization of single antibody–antigen recognition events and for the localization of antigenic sites. Perspectives of the method are discussed.

METHODS

Preparation of Sensor and Probe. A newly synthesized 8-nm-long polyethylene glycol (PEG) derivative (12) with an aminolinkable end and a thiol-reactive end was used as spacer for covalent linkage of the antibody to silicon nitride tips (Digital Instruments, Santa Barbara, CA) and of the antigen to mica surfaces (Mikrotechnik, Munich). Antibody (human serum albumin, HSA) and antibody (affinity-purified polyclonal anti-HSA antibody) were gifts from Boehringer Mannheim. Tips were cleaned by a standard procedure (13) for 10 min in chloroform and for 30 min in H2SO4/H2O2 (3:1 vol/vol), and then extensively rinsed with deionized water. Surface-bound water was removed by drying freshly cleaved mica and cleaned tips in an oven for 2 h at 180°C. For functionalization with amine-containing groups, tips and mica sheets were then immediately esterified (14) in a 55% (wt/vol) solution of ethanolamine chloride (Sigma) in dimethyl sulfoxide, overnight at 100°C with 0.3-nm molecular sieve beads while applying an aspirator vacuum and trapping H2O in a CaCl2 tower. Binding of PEG to amine-containing substrates and consecutive coupling of antibody and antigen to the thiol-reactive end of the spacer was done as described (12).

Determination of Surface Density. Sensitive high-resolution fluorescence imaging with accurate calibration (15, 16) was employed for the determination of the surface density of HSA and antibody, which had been fluorescence-labeled (12) prior to surface linkage. Probes were prepared with HSA densities between 100 and 1500 molecules per μm2 and sensors with antibody densities between 200 and 800 molecules per μm2. The latter values correspond to 0.5 and 2 antibodies per area of the tip end (2500 nm2, as estimated from the nominal tip radius of 50 nm). Probes with high HSA densities (1500 molecules per μm2) showed considerable clustering in fluorescence images.

Forces Microscopy. Cantilevers of sensors had spring constants between 0.11 and 0.27 N/m, determined as described (7). Briefly, a silicon cantilever (Digital Instruments, Santa Barbara, CA) with a spring constant of k0 = 0.18 ± 0.02 N/m was taken as reference lever. The silicon nitride cantilevers used in the study were calibrated by comparing their repulsive

Abbreviations: HSA, human serum albumin; SFM, scanning force microscopy.

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force–distance slopes on a solid support with slopes found in contact with the reference lever. Spring constants $k_u$ were determined by using the relation $k_u = k_d(s_d/s_r - 1)$, where $s_d$ and $s_r$ are the slopes measured on a solid support and on the reference lever, respectively. All silicon nitride cantilevers were calibrated with the same reference lever and calibrations were carried out in buffer. A Nanoscope III (Digital Instruments, Santa Barbara, CA) with a fluid cell, containing buffer [5 mM Hepes, pH 7.4/150 mM NaCl/lysolecithin (100 μg/ml)], was employed to record force–distance cycles at 1-Hz and 100-nm amplitude, if not stated otherwise. Adhesion forces between sensor tip and probe were generally absent. For applications of the sensor to biosurfaces, sensors showed no adhesion to a cell membrane (mast cell) or to a lipid membrane (dimyristoylphosphatidylcholine) on mica in aqueous solution with 150 mM NaCl.

RESULTS AND DISCUSSION

Detection of Antibody–Antigen Recognition Events. For a sensor molecule on the SFM tip, we chose a polyclonal anti-HSA antibody. The antibody was covalently linked to the tip via a flexible 8-nm-long PEG spacer that was newly synthesized for this purpose (12). The density of antibodies on tips was adjusted to best meet the expectation that only one antibody may interact with the probe. More specifically, the SFM tips carried many antibodies. Their surface density was, however, chosen sufficiently low so that, on the average, only about one of the flexibly linked antibodies is expected to be bound to the tip end that will reach to HSA molecules on the probe surface. The probe was HSA bound to mica with the same PEG spacer and allowed us to adjust the HSA surface density. Forces between sensors and probes with high HSA surface density (1500 molecules per μm²) were monitored during force–distance cycles (illustrated in Fig. 1A) by moving the probe continuously up (“trace”) and down (“retrace”) at constant lateral positions. Fig. 1 presents evidence that the sensor permits detection of single antibody–antigen binding events. Retraces of force–distance cycles showed attractive force signals of unique shape (Fig. 1A), interpreted to reflect antibody–antigen recognition. The attractive force develops nonlinearly with a significant delay as expected from stretching the long and flexible tip–probe connection after antibody–antigen association (for details, see Fig. 1A). The connection sustains the increasing force until the complex dissociates at a characteristic “unbinding force,” $f_u$. Such signals were repeatedly observed in consecutive force–distance cycles. Typically, 700 cycles were recorded at one fixed lateral position for which the 50 retraces shown in Fig. 1B are representative. Twenty-five of the retraces show one or two unbinding events. In the other cycles, there was no event detectable and retraces were identical to traces. For control, free HSA was washed, which effectively eliminated unbinding events (see control in Fig. 1B). Apparently, free HSA binds to the antibody, thus preventing recognition of surface-bound HSA. After washing out free HSA, unbinding events reappeared with a recovery time of 25 ± 5 min, determined for two sensors. One experiment is shown in Fig. 1C. The binding probability, $p_b$, which is the

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Fig. 1. Detection of single antigen recognition events by a tip–antibody sensor. (A) Effect of antibody–antigen binding on the force between tip and probe, illustrated for a typical record of a force–distance cycle. Binding of the antibody to the antigen during approach (trace points 1 to 5) connects tip to probe. This causes an extra force signal of particular shape (points 6 to 7) during tip retraction (“retrace”), reflecting extension of the flexible connection (point 6) to its full length. The force increases until unbinding occurs at the unbinding force $f_u$ (points 7 to 2). The length of the connection at the moment of unbinding, referred to as sensor length $l_u$, was determined by subtracting tip deflection from the distance between contact (point 4) and unbinding (point 2). Values of $l_u$ are predicted to be below 30 nm, assuming sizes of 6 nm for HSA and 8 nm for the antibody and each of the two PEG spacers. In calculations using the known isomerization potentials of PEG (17), the PEG chain was found to be extended to 95% at a force of 100 pN (ends free to rotate) with a similar nonlinear force–length profile as observed in unbinding events. The signal shown has $l_u = 19$ nm and $f_u = 268$ pN. (B) Force records of consecutive force–distance cycles. Unbinding events, shown here as upward signals, occurred in 25 out of the 50 records (only retraces are shown). The sensor had a spring constant of 0.27 N/m. Addition of 0.15 μM free HSA blocked binding of the antibody to surface-linked HSA (control). (C) Time profile of antibody–antigen unbinding events for block by free HSA and for its recovery upon HSA removal. The probability per force–distance cycle for antibody binding to surface-bound HSA was $p_b = 0.49$ (0–30 min). Injection of 0.15 μM free HSA resulted in a sudden and effective decrease of $p_b$ to 0.06. After 30 min, the probe was washed for 1 min with buffer after which unbinding events fully recovered within 27 min. Bars indicate times of continuous recordings of force–distance cycles at 1 Hz and their heights show the mean $p_b$ values observed.
FIG. 2. Characterization of antibody–antigen interaction by analysis of unbinding force \( f_u \) and sensor length \( l_u \). One-hundred fifty unbinding events were analyzed, detected in 201 force–distance cycles of a typical experiment. The sensor had a spring constant of 0.27 N/m. A step detection algorithm (18) was used to determine for each event \( f_u \) and \( l_u \) values and their uncertainties, which entered the probability density functions shown as Gaussians of normalized area. \( A \) Distribution of unbinding force. The \( f_u \) distribution has a maximum at \( f_u = 240 \) pN and a standard deviation of \( \sigma = 48 \) pN. The distribution of uncertainties in determining \( f_u \) values with a standard deviation of \( \sigma_u = 20.2 \) pN is included at zero force. The two peaks at higher forces reflect two unbinding events occurring at about \( 2 \times f_u \), which is attributed to simultaneous unbinding of the two Fab fragments. \( B \) Distribution of sensor length. The four retraces illustrate that unbinding events occurred at two distinct sensor lengths, yielding the \( l_u \) distribution shown.

Probability for observing an unbinding event in a force–distance cycle, was followed before and after addition of HSA and after its removal (wash). The high initial value of \( p_b \) is immediately reduced to about 10% upon addition of free HSA and fully recovered about 27 min after perfusion with buffer. The few events detected after addition of free HSA are attributed to adsorption of HSA molecules from solution to the probe surface. In additional controls using tips and probes with the PEG spacer bound but not conjugated with antibody and HSA, retraces were generally devoid of unspecific sensor–probe adhesion forces (retraces and traces were identical). Small adhesion forces were resolvable in less than 1% of the retraces that were easily discernible from unbinding events due to their clear separation from the delayed occurrence of unbinding. From controls and from observing unique signals of expected shape in the absence of adhesion forces, it appears safe to conclude that the signals are specifically due to antibody–antigen recognition. The ability of the antibody to bind, tested in more than 8000 force–distance cycles with four sensors, was found not to deteriorate, even after 2 months of storage in buffer.

Analysis of Unbinding Events. The force signal for unbinding contains information about antibody–antigen recognition at the level of single molecule interaction. This was addressed by analysis of the two signal characteristics at the moment of unbinding, the force \( f_u \) and the distance between tip and probe \( l_u \), referred to as sensor length (see Fig. 1A). Fig. 2 shows \( f_u \) and \( l_u \) distributions, determined from 201 consecutive force–distance cycles at one position on a probe at high HSA density (the 50 traces shown in Fig. 1B are part of these data). The values of unbinding forces \( f_u \) determined from retraces (see examples in Fig. 2B), resulted in a Gaussian-like distribution (Fig. 2A) with \( f_u = 240 \pm 48 \) pN (mean ± SD). Unbinding was detectable with a signal-to-noise ratio of \( f_u/\sigma_u = 12 \), where \( \sigma_u = 20.2 \) pN is the mean uncertainty in determining \( f_u \) values (see distribution at zero force). This unitary force is attributed to the recognition of single HSA molecules by one of the two binding sites of the antibody. Independent binding of the two Fab fragments of the antibody is evident from the frequency of unbinding events. The 201 retraces showed up to two events (Fig. 2B) with frequencies of 50, 100, and 51 for 2, 1, and 0 events per retrace, respectively. These data match a binomial distribution for the binding of two Fab fragments with equal probability of 0.5. Apparently, among the antibodies on the tip, only one had effective access to the surface-bound HSA molecules.

The distribution of sensor length \( l_u \) in Fig. 2B extends to values up to 30 nm, which conforms with an estimate for the maximal \( l_u \) value, assuming sizes of 6 nm for HSA and 8 nm for the antibody and each of the two spacers. The distribution is bimodal (maxima at 13 and 21 nm), indicating that the two Fab fragments unbind at different sensor lengths, which is attributed to an asymmetry introduced by the site of its coupling to

FIG. 3. Localization of antigen sites by a scanning tip–antibody sensor. \( A \) Histogram of unbinding events for the sensor passing one HSA molecule. The probe was laterally moved at 0.6 nm/s during force cycles at 3 Hz with a 100-nm amplitude. The number of events was sampled every 2.6 nm, corresponding to 13 cycles. The recognition profile represents 23 unbinding events in total. Mean distance between HSA molecules was \( \approx 100 \) nm, as determined by fluorescence microscopy. Statistical analysis showed that the peak position reflects the true mean of the distribution within an uncertainty of 1.5 nm. \( B \) Overlay of binding profiles as seen in \( A \). The distribution contains the data from six profiles, normalized to the average binding probability of 0.38, at maximum. It has a width of 6 nm, determined from the standard deviation of the Gaussian fit shown. All events occurred singly in force–distance cycles, indicating recognition of single HSA molecules. The mean \( f_u \) value was 270 pN for the chosen cycle rate of 3 Hz and cantilever spring constant of 0.22 N/m.
the spacer. Irrespective of the apparent difference in sensor configuration during unbinding, the two Fab fragments showed virtually identical unbinding force distributions (Fig. 2A Inset). Also, $l_A$ and $f_A$ values occurred in unbinding events without correlation (coefficient, $0 \pm 0.05$). Analysis of three sensors with significantly different $l_A$ distributions showed similar $f_A$ distributions and mean values within $f_A = 244 \pm 22$ pN. These findings indicate that the configuration of the molecular link and its momentary length during unbinding does not influence the unbinding force, attributed to the flexibility introduced by the spacer molecule. This provides confidence that the $f_A$ value is an unimpaired measure for antibody–antigen dissociation.

The described analysis was used to select sensors with effectively one antibody having excess to surface-bound antigens, selected by criterion of no more than two events per retrace. This applied to about 30% of the sensors in preparations at appropriate antibody surface density. For the other sensors up to six events were found in force–distance cycles, indicating that up to three antibodies were able to bind to HSA on the surface.

Localization of Antigenic Sites. The potential of the sensor for localizing individual antigenic sites was analyzed. For this, we prepared probes with a mean distance of $\sim 100$ nm between HSA molecules (an $\sim 15$ times lower density than was used for the analysis in Fig. 2). Unbinding events were detected as described above by moving the probe up and down in cycles of 3-Hz and 100-nm amplitude. During this vertical cycling, the probe was also moved horizontally with a constant velocity of 0.6 nm/sec, resulting in one force–distance cycle during a 0.2-nm lateral movement. As expected for this low HSA surface coverage, unbinding events in retraces during lateral line scans were found only at certain positions. Fig. 3A shows a typical profile of unbinding events from an individual HSA molecule. All 23 events in the profile occurred singly in retraces, indicating that only one HSA molecule was available for antibody binding. The position of the HSA molecule can be determined from the maximum of the profile with an accuracy of $\pm 1.5$ nm. Six such profiles for individual HSA molecules found are overlayed in Fig. 3B. The data match a Gaussian function with a mean width of 6.0 nm. Apparently, the 8-nm-long flexible spacer allowed the antibody to bind to an HSA molecule within a distance $r_{eff} = 6$ nm around the momentary position of the scanning tip. This dynamical reach enabled the antibody on the scanning sensor to bind during passing one HSA molecule about 20 times with probabilities as seen in Fig. 3B. This renders detection of antigenic sites reliable (probability for missing is $\sim 10^{-4}$). These findings indicate an appropriate choice of the spacer, which is sufficiently flexible and long for effective and fast recognition of antigens and sufficiently short for their accurate localization.

The flexibly linked antibody was not found to be entrapped between tip and probe since the points of tip–probe contact in force–distance cycles were as expected for a plain surface. This also indicates that the imaging capability of the tip is not significantly perturbed by the chemical treatment for antibody linkage. This opens the perspective for a microscopy capable of simultaneously imaging surface topography and the distribution of recognition sites or of mapping the molecular topography of biosurfaces. While the sensor appears apt for such uses, realization will require software extension of measuring modes to area scans during force–distance cycles at an appropriate feedback control that is currently being developed.\(^1\)

\(^1\)For the Nanoscope III used, lateral scans during force cycles were restricted to line scans, combining drift compensation with force calibration mode. From the data in Fig. 3, it is predicted that for an area scan at a 30 times faster lateral scan velocity (one cycle per $r_{eff} = 6$ nm), the probability for detecting antigens is 95% and an area scan of 0.1 x 0.1 $\mu$m$^2$ would last 90 s. For area scans with simulta-

\section*{Potentials for Studying Kinetics of Antibody–Antigen Interaction.} From the dynamical reach $r_{eff}$ and the vertical scan velocity, it is possible to estimate the antibody–antigen association rate constant $k_{ass}$. The antibody screens for binding within an effective volume $V_{eff} = 450$ nm$^3$ (half-sphere with radius $r_{eff}$). For a binding probability of 0.5 (found for a scan velocity of 200 nm/s, see Fig. 2), the time for which tip–probe distance is less than $r_{eff}$ is $t_{ass} = 2r_{eff}/200$ nm/sec $= 60$ ms. This yields $k_{ass} = N_A V_{eff} / t_{ass} = 5 \times 10^4$ M$^{-1}$s$^{-1}$, which is within the range of values typically found for polyclonal antibodies against peptide epitoles (19, 20). This confirms the conclusion that the antibody is quite free to move and orient for binding within the constraints set by its dynamic reach, which compares with the length of the PEG spacer. The recovery time from block by free HSA of 1500 s is a direct measure for the lifetime of the antibody–antigen complex in the absence of force, $\tau(o)$, or for its dissociation rate constant, $k_{diss}(o) = 1/\tau(o) = 6.7 \times 10^{-4}$ s$^{-1}$. This results in binding constants for the polyclonal antibody to HSA of $\sim 10$ nM, which is in agreement with values of 5–20 nM found in independent measurements (Boehringer Mannheim, personal communications). The potential of the method for determining rate constants of antibody–antigen interaction. The use of the method for quantitation of kinetic constants will require a careful calibration with monoclonal antibodies for which rate constants for binding to their antigens are well known.

A force acting on the antibody–antigen complex essentially reduces its lifetime. During a force–distance cycle, the force increases at a constant rate until the complex dissociates at force $f_A$. Therefore, the $f_A$ values are dependent on the rate of force increase, $df/dt = \text{vertical scan velocity times cantilever spring constant}$. An effective lifetime can be estimated (21) by the time the cantilever spends in the force window spanned by the standard deviation of the $f_A$ distribution. The time the force increases from $f_A - \sigma_f$ to $f_A + \sigma_f$ is then given by $\tau(f) \approx 2\sigma_f df/dt$, yielding 1.8 ms for the data in Fig. 2. Apparently, the lifetime is reduced by $8 \times 10^3$ times by the force of 240 pN compared to its value in the absence of force, $\tau(o)$. From this reduction, one may estimate an "effective rupture length $l_r"$, by using the relation $\tau(f) = \tau(o) \times \exp(-f_A/kT)$ (22). This yields $l_r \sim 0.23$ nm for the bonds involved in antibody–antigen interaction, which is within the expected range (6).

The accuracy and reliability in determining $f_A$ distributions may permit the use of the method for "force spectroscopy," i.e., for investigating relationships between unbinding force $f_A$ and lifetime $\tau(f)$ by applying various force rates $df/dt$ or particular force–time profiles. Such relations may well reflect slow structural relaxations of the molecular complex with characteristic times within the experimentally accessible range of 10 $\mu$s to 10 s.

\section*{CONCLUSIONS}

Flexible linkage of an antibody to a SFM tip has allowed the detection of single recognition events between an antibody and an antigen. Analysis of force profiles revealed insight into the process of antibody–antigen binding and unbinding at the level of single molecular events, unconstrained by the linkage used. Antigenic sites were reliably detected during lateral scans. This was rendered possible by realization of a sensor design with effectively one antibody covalently coupled to the tip via a sufficiently long flexible spacer molecule. The functional

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\includegraphics[width=\textwidth]{example.png}
\caption{Example figure for Biophysics: Hinterdorfer et al.}
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groups of the spacer used are applicable to coupling of ligands and proteins in general, which provides the method with a broad perspective in the study of molecular recognition. With the possibility of combining molecular recognition by ligands with structural resolution by the tip, a first tool comes into sight for molecular mapping the topography of biosurfaces.

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