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STUDY ON THE INTERACTION IN SINGLE ANTIGEN-ANTIBODY MOLECULES BY AFM

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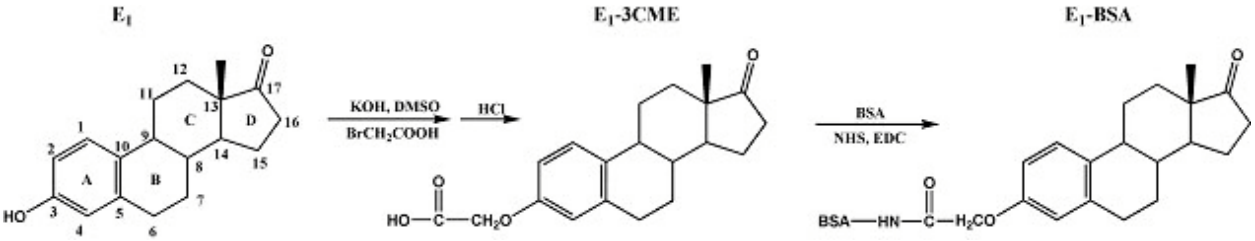
1. ABSTRACT

The interaction between single synthesized antigen and its monoclonal antibody was directly monitored by Atomic Force Microscopy (AFM) with antigen-labeled AFM tip. The details about tip modification and antibody immobilization were described. The interactions were monitored by the change of adhesive force in separating the bound antigen-antibody. The results showed that two processes could be observed in the combination of antigen-antibody system. Possible mechanisms of rupture process as independent unbinding and cooperative unbinding were discussed. At slow separation process, the antigen-antibody linkage appeared to rupture sequentially. Increasing the separation rate from 50 nm/s to 2000 nm/s led to an increase in the possibility of cooperative unbinding. The pH influenced the affinity constants greatly, but had little effect on the magnitude of unbinding forces. The obtained results were compared with affinity constants between complete antigen and antibody obtained by immunoassay method (ELISA). The observed single rupture force between individual antibody and antigen molecules provided pertinent information relating to the manner how the antibody molecule binds to its specific antigen.

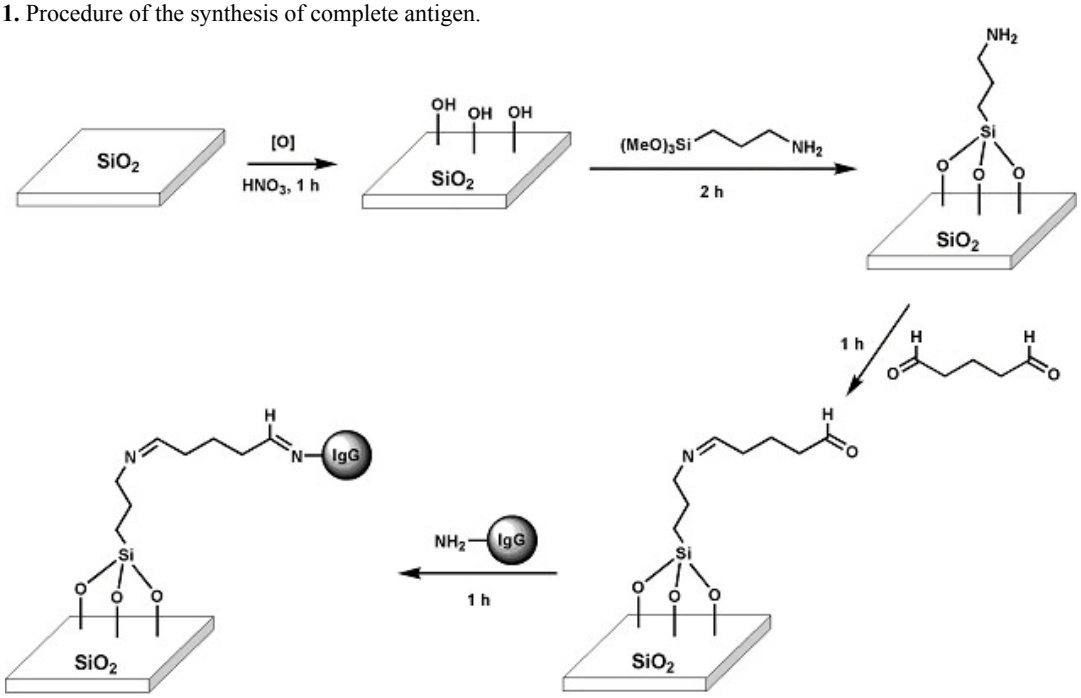
2. INTRODUCTION

Molecular recognition in biological system plays a pivotal role in nature in the events that regulate biochemical activity. In the simplest case, the chemical message is transmitted when the signal molecule, or ligand, binds to the correct receptor molecule – much like a key fits in the appropriate lock. The selectivity of the molecular interaction is fundamental to regulation of many biological processes. Some specific molecular recognition events, including molecular recognition between receptor and ligand (1, 2, 3), antibody and antigen (4, 5, 6, 7), and complementary strands of DNA (8, 9, 10), have been studied by various research groups by different techniques. The molecular structure is the key factor to how the receptor identifies the appropriate ligand in the process. Most protein receptors are designed to fit the ligand neatly into a "pocket" of its structure, where the functional groups of the ligand have specific points of the contact with functional groups of the receptor. For the correct molecular pair, these contacts serve as anchoring points to secure the ligand in the binding pocket. The antigen-antibody system is just such a kind of ligand-receptor system.

The interactions of antigen and antibody involve van der Waals interactions, hydrogen bonds, and, to a less extent, the formation of salt-bridges (11). The relative strength of the antigen-antibody interactions are usually estimated by affinity constants determined experimentally by immunoassays (12), surface plasmon resonance (13), and isothermal titration calorimetry (ITC) (14). However, all these methods provide information of integrated interaction behaviors but little about the force strength of the antigen-antibody interaction. The atomic force microscope (AFM) is a powerful tool for investigating the topographic images of samples surfaces as well as the forces (15). Its ability to directly measure discrete intermolecular force of 10 pN or less was first highlighted by Hoh *et al*. and later utilized on molecular recognition by Gaub's group in the streptavidin/biotin system (16, 17). Similar approaches have been reported measurement of biotin-avidin interaction. Stuart's group has investigated the specific interaction between an antifluorescyl antibody functionalized surface and fluoresceinated silica beads (18). Hinterdorfer and coworkers monitored the process of antibody-antigen binding and unbinding at the level of single molecular events successfully (4). Willemsen *et al*. obtained simultaneous height and adhesion imaging of antibody-antigen interactions with a modified AFM and the comparison between the height image with the adhesion



Scheme 1. Procedure of the synthesis of complete antigen.



Scheme 2. Reaction scheme for substrate preparation.

image showed a high correlation of specific molecular recognition with topography (19). In AFM measurements, possible conformational changes of proteins during their adsorption to surfaces may lead to a reduction in the number of sites where the receptor-ligand complex can form. In order to minimize the impact of different conformation that proteins may take, spacing molecules with lengths of several nanometers were used as flexible cross-linkers to enhance mobility and increase the binding possibility (5, 20). The other reliable way is to choose molecules with binding sites less dependent on the conformation or orientation of molecules on the probe surface. With ferritin-functionalized AFM probes, Allen *et al*. monitored the adhesive forces between the probe and antiferritin antibody-coated substrates and suggested the presence of periodic force attributed to single unbinding events between individual antigen and antibody molecules (6).

AFM was used to monitor the interaction force of an artificial antigen and its monoclonal antibody system, E_1-3-hemisuccinyl-bovine serum albumin (E_1-BSA), and the monoclonal antibody in this paper. The experiment was performed in aqueous physiological solutions, under which condition the activity of the antigen and the antibody could be preserved. The molecular ratio was analyzed as 1:28 (protein:estrone). Force measurement was achieved by first approaching the tip to the substrate surface to ligate the E_1-BSA on AFM tips to the IgG antibodies on substrates and then pulling the complexes until the interaction bonds broken. Their magnitudes were compared at different separation rates and pH. The results obtained from this technique was compared with that obtained by ELISA.

3. MATERIALS AND METHODS

3.1. Complete antigen synthesis and monoclonal antibody purification

Estrone(E_1) a small molecule with molecular weight of 270, is a hapten that can not induce immune response and thus BSA was used as a carrier to synthesize the complete antigen or the immunogen. Complete antigen of estrone was synthesized with BSA using the method reported by Wang (21) (Scheme 1). Though it may lose some antigenic by the variation in the nature of substitutes in the A-ring (C-3-OH), the anti-E_1 antibody with least cross-reactivity can be raised by exposing the unique functional group in the D-ring. Andrieu *et al*. demonstrated that highly specific antigera for E_1 could be obtained by employing E_1-3-hemisuccinyl-BSA conjugate (22). A coupling ratio of 28 estrone residues per protein molecule was determined. Estrone monoclonal antibody was purified from mouse ascites fluid according to the method described before (23).

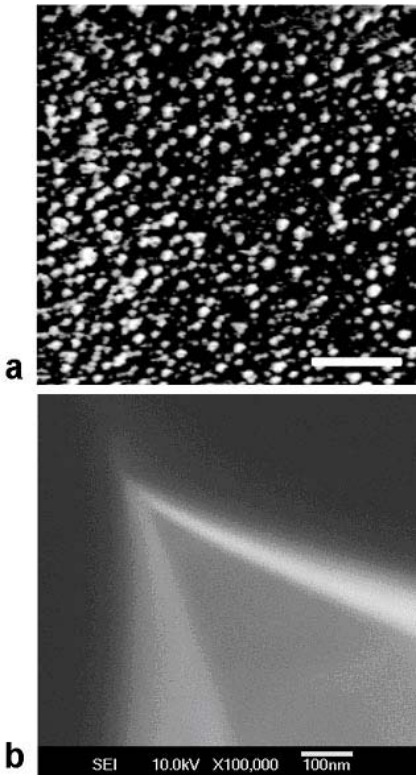


Figure 1. a) The topographic image of immobilized protein molecules acquired in tapping mode. b) HRSEM image of the tip of a silicon nitride (Si_3N_4) probe. Minimal scales represent 200 nm and 100 nm, respectively.

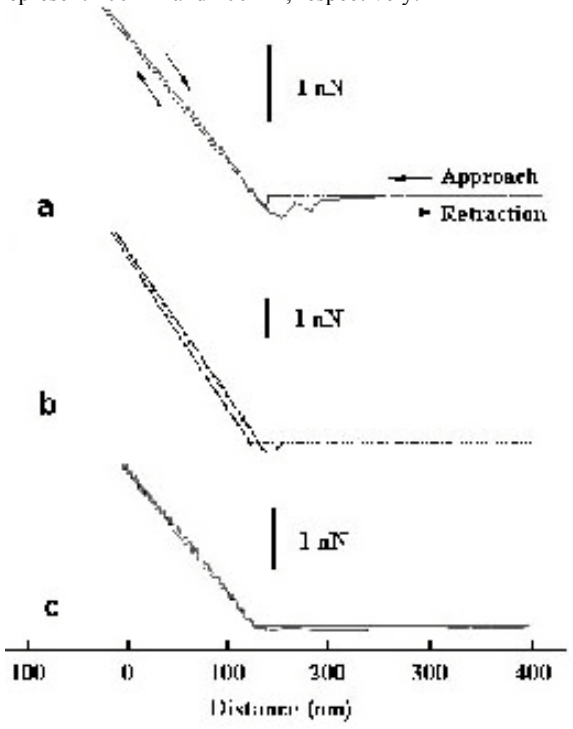


Figure 2. a, b) Typical force-distance curves of approach/retract cycles taken between E_1-BSA-coated probe and an IgG functionalized substrate; c) control: the force-distance curve taken after blocking the binding sites with free estrone.

3.2. Preparation of antibody-modified substrate

Typically, the slides were first cleaned thoroughly by dipping into 1% NaOH aqueous solution and followed by 3% HCl aqueous solution both at 90□ for 10 min. Then placed them in boiling 35% HNO_3 for another 1 h and successively washed several times with distilled water and methanol before dried under vacuum overnight (24). The dried slides were dipped in 1.5% v/v solution of 3-aminopropyltriethoxysilane in toluene for 2 h and afterwards sonicated in the series of solvent to remove any unbound silane. The silanized slides were further placed in a 10% v/v solution of glutaraldehyde (AR, 25% in aqueous solution) in potassium phosphate buffer (100mM, pH 7) for incubation at room temperature for 1 h. The activated wafers were subsequently rinsed with deionized water to remove any unreacted glutaraldehyde before the antibody solution (1.5 mg mL^{-1} in phosphate buffer) was dropped onto the surface of each slide for another incubation of 1 h at room temperature. Finally the modified slides were sufficiently rinsed with distilled water and buffer to remove any loosely bound biological materials and stored at 4□ in phosphate buffer until used (6).

3.3. Probe functionalization

Cantilevers (Nanoworld, Swiss) with sharp silicon nitride (Si_3N_4) tips were functionalized with complete antigen of E_1-BSA via the following immobilization protocol: 50 µl of complete antigen at 1mg/ml was adsorbed onto the tip overnight at 37□. Then the cantilevers were consecutively rinsed with phosphate-buffered saline (10mM, pH 7.2), distilled water and also stored in buffer at 4□ until used (2). The image of the tip was obtained with a JEOL JSM 6700F field emission high-resolution scanning electron microscope.

3.4. Force measurement

Force scan measurements were recorded with a SPA300 AFM (Seiko, Japan) and a scanner of 20µm×20µm was applied. The topographic images of the protein molecules were obtained by scanning the substrate in tapping mode under the buffer solution. Force-distance curves were acquired at designated points in roughly prescanned areas with a scanning frequency of 0.5Hz and then directly export from the AFM.

3.5. Determination of affinity constant (K_{aff}) of anti-estrone antibody

The method was derived and adapted from that of Beatty *et.al*. (25), in which serial diluted antigen, coated onto the microtiter plates, and antibody were applied to measure K_{aff} using the Law of Mass Action. The amount of antibody bound to the coated antigen was represented as a sigmoid curve of OD vs. the logarithm of antibody concentration added to each well. Buffer was made by KH_2PO_4 and Na_2HPO_4 solution with pH values ranged from 4.95 to 8.91. Immediately after the color development, the plates were put into the detection chamber of an automatic microplate reader (Tecan, Spectra Rainbow) and shaken for 15 sec. The measurement was conducted with absorbance and reference at wavelengths of 450 and 492nm, respectively (26).

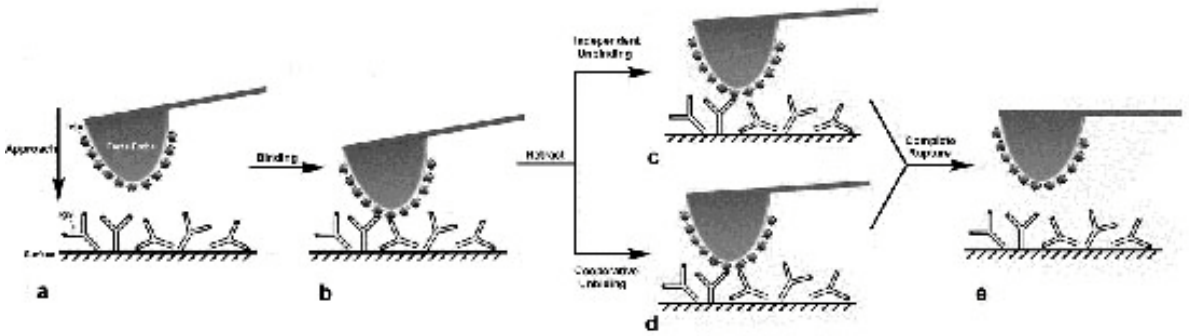


Figure 3. Schematic of possible mechanisms for detachment of antigen-antibody pairs: a) Approaching of the force probe to the antibodies; b) specific binding of two antigen-antibody pairs; c) During independent unbinding, bonds rupture sequentially with a small detachment force; d) Unbinding in a cooperative way, bonds break synchronously and the detachment force is much larger; e) Complete rupture of the bindings between antigens and antibodies.

K_{aff} was calculated by the following equation:

$$K_{aff} = (n-1)/\{2(n[Ab']-[Ab])\}$$

Where $n = [Ag]_t / [Ag']_t$ ($[Ag]_t$ and $[Ag']_t$ are two different coated antigen concentrations), $[Ab']_t$ and $[Ab]_t$ represent the measurable antibody concentrations at OD-50 (50% of the maximum value) and OD-50' for plates coated with $[Ag]_t$ and $[Ag']_t$.

4. RESULTS AND DISCUSSION

4.1. Substrate and tip

We have tried several kinds of substrate as monocrystal silicon, gold wafers and glass slides processed with different methods. Glass slides were found to be the best for this purpose: due to their proper reactivity, it is possible to couple the proteins to the substrates at a uniform density with good reproducibility. The detailed treating of the glass slides has been discussed in Materials and Methods section and can be summarized as scheme 2. It has been reported that covalent bond could withstand forces up to several nanonewton (27), which is far beyond the maximum force in our experiments, thus proteins were covalently immobilized to avoid its rupture from the glass slides during the measurement.

Figure 1a shows a typical topographic image of the modified substrate. The proteins attached to the substrate were fairly monodisperse with a diameter of 21 ± 3 nm. Figure 1b is the high-resolution scanning electron microscope (HRSEM) image of the probe and the diameter of the sharp tip was estimated as around 20 nm, a size comparable to that of the protein. Thus in most cases, the effective contact area of the probe was small enough for BSA molecules absorbed on the tip to contact and interact with only one IgG antibody, which led to the observation of unbinding events between them.

4.2. Force measurement

It was reported that the separation rate could influence the unbinding force to some extent(28). With the increasing of the separation rate, the unbinding would also increased, but for different zone of separation rate, the increasing ratio (increasing force VS increasing separation rate) was also different. Furthermore, for different system, the zone was also different. In our experiment, with the increasing of the separation rate (from 50 nm/s to 2000 nm/s), the force of single unbinding force did not increase significantly, only about 20pN for the average value. But when the separation rate reached 4000 nm/s the force became about 45 pN more than the value of 2000 nm/s, the increasing ratio increased more than two times. So in the zone from 50 nm/s to 2000 nm/s, where the separation rate was relatively low, the increasing ratio was also relatively low. In this zone, another new phenomenon was noticed, at the separation rate of 200 nm/s, the second force peak appeared, and with the increasing of the separation rate, at 2000 nm/s, this force peak became higher. This phenomenon was accompanied with the change of the original force-distance graph. Typical force-distance curves of approach/retract cycles were demonstrated in figure 2. Over 80% of the curves showed two unbinding events in the retraction process as given in figure 2a, while all other curves like figure 2b showed single unbinding events with about twice in the rapture force. It should be noted that all curves obtained at low separation rate exhibited two unbinding events, indicating that the measured adhesion forces were mediated by double binding events of antibody-antigen interactions. In order to validate the biological specificity of the antigen-antibody interaction, a series of experiments were performed: Free estrone was added to the solution to block the binding site of the antibody on the substrates. As a control, no adhesion force was observed this time as shown in figure 2c. Moreover, the ELISA results between the blocked and unblocked wells also gave evidence that there was little non-specific adsorption, as might be expected from the fact that the fused cells had been filtered and only the cells which had least unspecific adsorption.were left. Thus the force measured was the unbinding force of the specific binding site between antigen and antibody without any portion of unspecific adhesion force.

Figure 3 depicted possible mechanisms for detachment of antigen-antibody pairs. Upon approaching the force probe to the antibodies, double specific bindings between antigens and antibodies were formed (figure 3a and 3b). Since the measured adhesion involves the cooperative properties of the double bindings, the dynamic

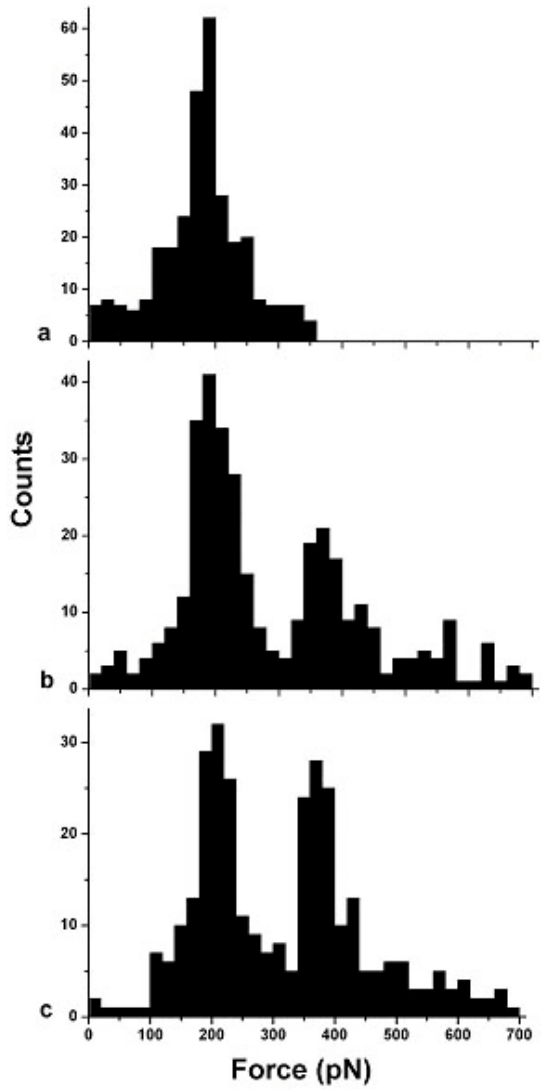


Figure 4. Force histograms of the antigen-antibody interaction obtained at different separation rates of a) 50, b) 200 and c) 2000 nm/s.

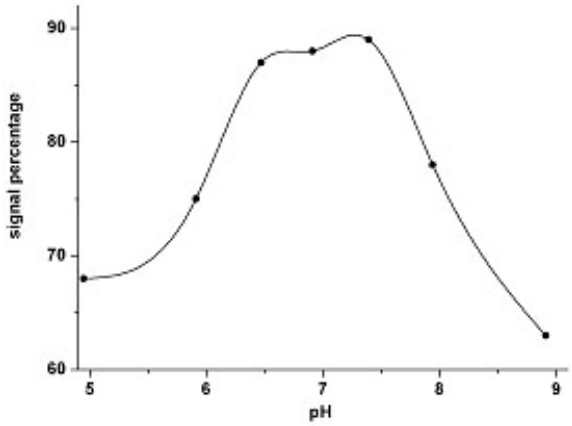


Figure 5. The signal percentage of force measurement at different pH. The separation rate of the measurement was 2000 nm/s.

response during separation process can be categorized as following two ways: If the applied force is distributed unequally between the bonds, the two bonds will rupture at different times as shown in figure 3c and two resolved peaks appear in the force-distance curves of figure 2a; On the other hand, if the applied force is distributed evenly on both bonds, they will dissociate simultaneously in a cooperative way with a larger detachment force (figure 3d) and only a single rupture can be seen as in figure 2b. Sufficient retraction leads to complete rupture of any specific bindings of antigen-antibody pairs in the end (figure 3e).

To examine the effect of experimental conditions on detachment mechanisms, the unbinding of antigen-antibody bonds were characterized at different rates of cantilever retraction. Histograms of rupture force at different separation rates of 50, 200 and 2000 nm/s were obtained from the study of the force measurement process (figure 4). At a separation rate of 50 nm/s, all force measurements showed two distinct unbinding events in force-distance curves, representing the hundred-percent presence of independent unbinding therein. The majority of rupture forces were distributed in the range of 80-280 pN and clustered in a single distribution as shown in figure 4a. Gaussian simulation of the force distribution suggested the rupture force for a single antigen-antibody pair was 183 ± 19 pN, a value consistent with previous results for similar experiments between antigens and IgG antibodies varying from 60 to 244 pN (4, 5, 18). At the separation rate of 200nm/s (figure 4b), a second force distribution centering at *ca.* 370 pN became evident, which was about twice of the single separation force for single antigen-antibody pair and attributed to synchronous rupture of the double bindings corresponding to cooperative unbinding events. Increasing separation rate to 2000 nm/s in figure 4c further augmented the possibility of cooperative unbinding. Combining all the data presented in figure 4, the force histograms revealed that there was a shift toward greater cooperative unbinding with increasing separation rate as predicted by the Bell model (29). This was a natural result, because at higher separation rates the two bindings may have less time to interact and achieve the equilibrium, a preliminary requirement for sequential ruptures as in the case of independent unbinding. We also notice the occurrence of forces larger than 500 pN in the force histograms. Since the formation of three or more firm binding is very difficult in our conditions, these should be a summation of ruptures from several unorientational bindings.

To investigate how the rupture process is modulated by changes of ligand affinity, the adhesive forces of antigen-antibody were measured over the pH range of 5-9. Table 1 summarizes the apparent affinity constant of monoclonal antibody with the complete antigen at different pH values. K_{aff} varied by approximately three orders of magnitude in the regime and maximized at pH 7.4 with a value of 5×10^8 L/mol. The lower binding affinity at other pH values reflected a reduced antibody population that was capable of binding to antigen. In force measurement, this reduction was presented as the decreasing counts of antibody molecules that could engender rupture signals in the retraction section of force-distance curves. Figure 5

Table 1. Affinity constant at different pH values

pH	4.95	5.91	6.47	6.91	7.39	7.94	8.91
$OD_{,max}$	0.9136	0.9428	1.004	1.001	1.0015	0.9332	0.8534
K_{aff}	6.7×10^6	8.9×10^6	2.7×10^8	4.2×10^8	5.0×10^8	6.4×10^6	7.9×10^5

outlines the signal percentage in force measurement and its variation was consistent with changes of K_{aff}. Interestingly, we detected little difference in the magnitude of unbinding forces at different pH values. These phenomena can be attributed to the alterations in the existing style of some amino acid residue in antibody molecules with pH variation. As a result, some of the antibody molecules lost the epitops or the binding sites, which induced the decrease in signal percentage. But for combined antigen-antibody pairs, the functional group of the binding site and the steric conformation remained unchanged, leading to the invariability in unbinding force.

5. CONCLUSION

In our experiment, artificial antigen was used instead of natural antigen. Compared with natural antigen, E_1-BSA has the following advantages: First, rigor in its structure ensures that the protein is composed of about 28 homologous subunits arranged in a spherical shell-like manner and contains many identical antibody binding sites or epitopes regardless of its conformation (see discussion section). Second, the versatility of synthetic antigen can be manipulated via organic synthesis and this paves the way for further investigating the difference among the manners that various small molecules interact with the specific biological macromolecule.

The results showed that the specific interaction between estrone complete antigen, E_1-BSA, and monoclonal antibody could be measured by AFM with a sharp tip. The adhesion force between the antigens and antibodies were primarily mediated by double bindings in this system and the detachment force for a single antigen-antibody binding was about 183 ± 19 pN. Control experiments and ELISA results proved the specificity of the adhesive forces. Besides sequential rupture of the bindings known as independent unbinding in retraction process, cooperative unbinding events were existent and favored at high separation rates. The pH value has great influence on the affinity constant, but little effect upon the unbinding forces of the antigen-antibody pairs. In the future research, present facile method can be applied to detect the distribution of the antibody molecules which probably exist on the membrane of the hybridoma cells or lymphocytes before the antibody molecules were secreted out of the cells.

6. ACKNOWLEDGEMENT

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