Force Measurement of Specific Antibody-Antigen Interactions in pH-varied Liquid Environments

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Abstract

The study reports the atomic force microscopy to measure the unbinding force of single antibody-antigen pair. The force measurements were conducted with the tip functionalized with antigens to its corresponding contact surface of the glass substrate functionalized with antibodies in aqueous solutions. In the approach and retraction of the AFM operation procedure, the contact force is greatly related, which occurs in retraction step. The contact forces are involved regular adhesion, nonspecific and specific bindings. Of great importance is the protein specific binding that demonstrates the shift of a slope in retraction step in apparent contrast to the nonspecific binding. The shifted sharp pull-off curve is attributed to the prior elongation of protein pair under external stretching and subsequent rupture of their bindings. the greatest unbinding forces were found in a range of nearly pH 7, indicating the value of 256.4 ± 48.9 pN at pulling velocity of 166.7 nm/s. A sharp decrease of the unbinding force occurs below pH 6.7, and a gradual decrease of environment found beyond pH 8.0. This is the first time that single human IgG1 and anti-human IgG1 pair interaction was quantitatively measured under the liquid environment from pH 2.0 to pH 10.0. The results are significant and provide direct unbinding force evidences in nearly realistic environments.

I. INTRODUCTION

The atomic force microscopy (AFM) [1] is a powerful tool to directly measure the force required to separate the intermolecular interactions in a scale of single bio-molecule pair. Use of the AFM technique exhibits several advantages that make it an optimum tool for measuring the intermolecular forces such as high force-sensitivity on the order of 10^{-14} Newton, vertical displacement sensitivity of 0.01nm, and ability to operate under liquid environment. Apparently, this technology allows us to study the strength of chemical bonds [2-4], force interactions between bio-molecules [5-15], and unfolding force of intermolecular interactions [16-19]. In those applications, as one interacting partner is to be immobilized on the AFM cantilever tip, interaction with the other specific samples on the substrate surface is probed with force curves. When samples of an AFM tip are retracted from a substrate surface, the unbinding force, defined as the maximum force at the moment of samples separation, can be

recorded and measured of the interaction between bio-molecules.

Several direct force measurement schemes of antibody-antigen interactions have been reported using different biophysical technology, including biomembrane force probe (BFP)[15], atomic force microscopy, and laser tweezers (LT)[20]. Specifically, the AFM and BFP techniques have been employed to characterize the unbinding force of single antibody-antigen complexes. The AFM exhibits great advantages in high resolution of force and ease of sample preparation. In this study, the AFM technique is chosen to investigate bio-molecular interactions.

In biomolecular samples, immunoglobulin G (IgG) is well popular and chosen in study of antigen-antibody interaction. The structure of human immunoglobulin G1 (IgG1) is constituted by four polypeptide chains which are connected by disulphide bonds and non-covalent bonds. The four polypeptide chains are entwined together in different fragments including two identical Fab segments and one Fc segment, as shown in Fig. 1. The IgG1 is in a Y-shaped formation. The binding sites of human IgG1 are located at the far end of the Fc segment [21]. The specific interaction between human IgG1 and anti-human IgG1 can be quantitatively described as the binding affinity. The affinity is measured by ELISA [22] to be around 1.7×10^{-9} M. In addition, the specific binding can also be distinguished by the parameter of surface roughness using AFM image. For specific interaction, the height of antibodies adsorbed onto mica and specific bound to the antigens is higher than those antibodies and antigens non-specifically adsorbed onto the mica surface. This approach also provides an alternative to discriminate specific and non-specific interaction between antibody and antigen [23].

This paper focuses on using the AFM as a biosensor to directly measure the biomolecular force interactions of human IgG1 (antigen) and anti-human IgG1 (antibody) in a liquid environment. The force measurement is conducted in pH-varied solutions to simulate protein live activity and the binding interaction. This study demonstrates the correlation of protein intermolecular unbinding force in various pH environments, and provides direct evidence of biophysical phenomenon.

II. MATERIAL AND METHOD

A. Functionalization of AFM Tips and Substrates.

Surface modification of the AFM cantilever tip (Nanosensors, Germany) and glass (Superfrost, Germany) is a key step in the successful investigation of biomolecular interaction by AFM force measurements. Biomolecular interaction studies were performed using AFM tips functionalized with human IgG1 and modified glass surface for anti-human IgG1. First of all, the modification of AFM tips and glass were carried out for surface cleaning in $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ (70:30, vol/vol) for 30 minutes, and then extensively rinsed with deionized water. The tips and glasses were then silanized with a 5% solution of 3-aminopropyltriethoxysilane (Fluka Chemie, Switzerland) in 5% ethanol solution at room temperature for 30min. The tips and glasses were then rinsed with solution A (5% ethanol / 95% deionized water). Third, the tips and substrates were immersed in a 2.5% glutaraldehyde solution in phosphate-buffered saline (PBS, pH 7.2) for 1 hour and then extensively rinsed with solution A. At last, the human IgG1 (100g/mL in PBS, pH 7.2) covalently binds to the AFM tip via their amino groups after incubation in PBS (pH 7.2) overnight at 4°C. The same process was done for anti-human IgG1 (100 g/mL) to the glass substrate. [5].

B. Calibration of Cantilever Spring Constant

All cantilevers require individual calibration of each cantilever used for quantitative measurement for large variations in mechanical spring constant of commercial silicon nitride cantilevers. Each cantilever was individually calibrated by thermal fluctuation method [24] to investigate its spring constant. The average spring constant for cantilevers employed in this experiment was measured around 80 ± 10 pN/nm. The sensitivity of individual cantilever also was calibrated by adopting the straight slope in plot upon the cantilever onto a concrete substrate surface. The sensitivity was obtained in the range of 11.4 mV/nm to 18.3 mV/nm by converting electrical signal with respect to cantilever distance (nm).

C. AFM Force Measurements and Data Analysis

The force measurements were performed with an SPA-300HV atomic force microscopy (SEIKO Instruments Inc., Chiba, Japan) equipped with a commercial liquid cell. The experiments were carried out in the liquid cell filled with freshly prepared aqueous solutions at room temperature. The signal of interaction between human IgG1 and anti-human IgG1 were obtained by recording the AFM cantilever and piezo-scanner position in a force curve cycle. Meanwhile, the electrical signal of cantilever deflection (mV) was first converted to its corresponding deflection distance (nm) which may then derive the corresponding force (Newton) using the abovementioned cantilever spring constant. Figure 2 shows a full curve cycle in approaching, touch-down, push-down and retraction process. In the retraction process, a sharp pull-off in

a force curve is found, indicating a sudden separation of the AFM tip out of its substrate surface. This might be attributed to contact surface adhesion or intermolecular unbinding force. In the case of a full force curve measured in a liquid environment, the adhesion force between both contact surfaces is nearly negligible. As a result in this study, the sharp pull-off signal is dominated with the unbinding force between antibody and antigen. Of special interest are the unbinding forces measured in various pH liquid environments to simulate protein interaction in live activities. The measurement was conducted in a constant speed of 166.7 nm/s during the course of approach and retraction, because change in speed may vary the values of unbinding forces. The results were obtained at all specific unbinding events recorded at least 100 individual unbinding force of each pH-specific environment. The analysis of statistic histograms with Gaussian fitting was made to demonstrate its range of unbinding forces [12-14].

Fig.1 The typical IgG1 molecule is constituted by four polypeptide chains which are connected by disulphide bonds and non-covalent bonds. The four polypeptide chains are entwined together in different fragments including two identical F_{ab} segments and one F_c segment which the form of IgG1 is a Y-shaped formation. The binding sites of human IgG1 are location on the far end of the F_c segment which is specific recognize to F_{ab} of anti-human IgG1.

III.RESULTS AND DISCUSSIONS

In order to understand the correlation of protein bond strengths in different pH liquid environment, the AFM technique is employed as a force-based biosensor to separate antigen-antibody and measure the unbinding forces. A schematic diagram of the protein force interaction between human IgG1 and anti-human IgG1 is shown in Figure 2.

Fig. 2. A schematic diagram of AFM as a biosensor to measure the force required to separate the human IgG1 and anti-human IgG1. The relative position of the sample surface to the AFM tip is indicated by the arrowheads. As the substrate surface approach to AFM tip from A to B, the cantilever is pulled down by the attractive force and jump-to-contact with the surface at B. As cantilever continues approaching to sample, the cantilever bends upward until reaches point C. When the tip reaches point 3, the cantilever begins to retract. As cantilever continues to retract, the cantilever is bended downward by unbinding force of human IgG1 and anti-human IgG1, until tip reaching break point D. The force increases until unbinding occurs at the unbinding force F . Finally, the cantilever returns to non-force state at point A.

It is noted that a sharp pull-off curve in a force curve is involved with adhesive interactions, specific antibody-antigen interactions and nonspecific interactions. As described earlier, the protein specific binding was greatly interested in various pH-value environments. The distinction between those forces involved is required. First of all, when the AFM tip with no protein was operated in liquid in a cycle of approach and retraction process, the results were recorded as a reference to the subsequent experiment. No apparently sudden pull-off in a force curve was found in a liquid environment where the van der Waals and viscosity forces were negligible. Furthermore, nonspecific binding experiment was conducted to discriminate specific and nonspecific binding between proteins. As an excess of antigens were flowed into the glass substrate which was covalently bound with dilute antibodies, an enormous fraction of the surface occurred in nonspecific binding. The AFM tip functionalized with linkers to effectively capture proteins approached down to the substrate in aqueous solution. In this experiment, a sharp pull-off curve occurred in the retraction. The nonspecific binding was mostly found when the attached proteins on a substrate was departed out of its surface, indicating an apparent adhesion force. The adhesion was attributed to the contact of the

linker-coated tip surface and glass substrate [10-11]. In comparison to the nonspecific binding, the subsequent experiment was conducted to investigate the discrimination between specific and nonspecific bindings. As the AFM tip covalently attached with antibodies was operated in the same process to its counterpart antigens on a substrate, a sharp curve was obviously found in retraction step as shown in Figure 3. The different of the specific binding to the nonspecific binding was apparently found in a shift of adhesion. The shift occurs in retraction step because the human IgG1 and anti-IgG1 pair in specific binding is elongated under stretching in the beginning, and then suddenly ruptured in pair separation. The monoclonal antigen and antibody was single paired, as illustrated in Figure 3 (a) or (b). Figure 3(c) shows its corresponding force in shifted sharp pull-off curve. In conclusion, the specific and nonspecific protein interactions can be discriminated in a force curve by the shift in slope during the retraction step. The force measured in specific binding of single antibody-antigen pair also indicates the unbinding force (F).

As described the single sharp pull-off curve, the sequential double events were also found in experiments.

Fig. 3. The schematic diagram shows the force curve of single unbinding event corresponding to probable binding position of human IgG1 and anti-human IgG1. The anti-human IgG1 immobilized could be in position of (a) standing or (b) lying on the substrate in which the specific binding occurs between proteins. (c) A typical force curve of single human IgG1 vs. anti-human IgG1 is demonstrated.

Similarly, the double unbinding events are attributed to the interaction of antibody molecules to antigen human IgG1. The double unbinding points could correspond to the sequential unbinding of the Fab arms of one antibody from two biomolecules of human IgG1, or two biomolecules of human IgG1 ruptured out of two antibodies. In addition, the multiple sharp peaks of data could also be found in this study. As the environment is varied in pH conditions, the unbinding force may induce conformational transitions in the binding sites of protein and induced protein-unfolding events [12]. In other words, the multiple unbinding events measured may reflect to the issue of protein-unfolding which is not addressed in this paper.

To quantify the force required for human IgG1 and anti-human IgG1 to separate one single antibody-antigen pair, all specific unbinding events were recorded and plotted in histograms, which were subsequently analyzed in Gaussian fitting [12-14]. The histograms of specific force measurements were made in disparate pH solutions in value of 4, 6, 7, 8, and 10 at constant pulling velocity 166.7nm/s. The measured unbinding forces were collected over 100 unbinding events which were obtained from 500 to 800 approach/retraction operations. Meanwhile, each histogram was also made by two or three tips in which the averaged results may exclude effects from spring constants and protein density variation at the AFM tip or the glass surface.

The correlation of unbinding forces in pH-varied liquid environments has been investigated, simulating the protein interaction and activity in nearly realistic environment. Figure 4 shows the unbinding forces between human IgG1 and anti-human IgG1 in pH-varied environments. As demonstrated, the greatest unbinding forces were found in a range of nearly pH 7, indicating the value of 256.4 ± 48.9 pN at pulling velocity of 166.7 nm/s. A sharp decrease of the unbinding force occurs below pH 6.7, and a gradual decrease of environment found beyond pH 8.0.

As demonstrated in Figure 1, the F_c fragment of immunoglobulin is most sensitive to its surrounding lower pH

environments. The acidic environment may induce denaturation of secondary structures constituted by the low β -sheet contents such that the unbinding force of antibody-antigen complex was sharply decreased [25].

Fig. 4. The unbinding forces measured in various pH liquid environments exhibit the maximum unbinding force of 247.7 pN in a pH 7 solution at pulling velocity 166.7nm/s. A sharp decrease of the unbinding force occurs below pH 6.7, and a gradual decrease of environment found beyond pH 8.0.

IV.CONCLUSIONS

The atomic force microscopy as a force-based biosensor to measure the unbinding force of single antibody-antigen pair has been successfully demonstrated. The force measurements of tip functionalized with antigens and glass functionalized with antibodies were conducted in aqueous solutions. Those contact forces involved were carried out in discrimination between regular adhesion, nonspecific and specific bindings. The specific antibody-antigen binding shows the shift of a slope in retraction step in apparent contrast to the nonspecific binding. The shifted sharp pull-off curve is attributed to the prior elongation of protein pair under external stretching and subsequent rupture of their bindings. the greatest unbinding

forces were found in a range of nearly pH 7, indicating the value of 256.4 ± 48.9 pN at pulling velocity of 166.7 nm/s. A sharp decrease of the unbinding force occurs below pH 6.7, and a gradual decrease of environment found beyond pH 8.0.

This is the first time that the individual interaction force of single human IgG1 and anti-human IgG1 pair was quantitatively measured under the liquid environment from pH 2.0 to pH 10.0. The results are significant and provide direct unbinding force evidences in nearly realistic environments.

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