Chapter 5

Quantitative Analysis of the Number of Antigens Immobilized on a Glass Surface by AFM
5.1 Summary

To develop force measurements using an atomic force microscope (AFM) in a quantitative manner, it is necessary to estimate the number density of target molecules on a sample surface, and for this, the sensitivity of detection should be known. In this study, the AFM was used as a mechanical detector and an antigen and its antibody were used as a model to evaluate the sensitivity of detection. Antigens were immobilized on a glass surface and number density was estimated by monitoring optical absorbance due to product formation by the reaction of crosslinkers. The concentration of antigen was controlled by mixing control peptides. A microbead was used as a probe and antibodies were immobilized on the bead. AFM force measurements were then made for a range of number densities in the order of 10 to 10^6 antigen molecules per square micrometer of surface and were compared to evaluate the sensitivity of detection. Our result establishes the reliability of estimating numbers of molecules like receptors on the cell surface, and indicates that the AFM is useful as a mechanical detector with high sensitivity.
5.2 Introduction

In previous studies, we introduced a method of extending the measurement of cell adhesive interaction from the single molecular level to the cellular level. Cell adhesion is fundamental to many kinds of biological processes such as cellular migration and differentiation. Molecular recognition such as the detection of a differentiation signal is of critical importance to the regulation of such processes. To know the mechanisms of molecular recognition and the responses of cells to stimulation, amounts and distributions of receptors on cell surfaces are good parameters, because the expression of receptors should be controlled by the stimulation, and both the amount and distribution should be changed. To study the distribution of receptors on the cell surface using an AFM, a microbead was employed as a probe to increase the area of contact with the cell and the distribution of molecules such as integrins was observed on a living cell. However, to develop a more quantitative measurement, it is necessary to estimate number densities of target molecules on cell surfaces, and for this, the sensitivity of detection of the AFM should be known. In the present study, an AFM was used as a mechanical detector and an antigen and its antibody were used as a model to evaluate the sensitivity of detection. The result establishes the reliability of our method on estimating numbers of molecules like receptors on the cell surface, and indicates that the AFM is useful as a mechanical detector with high sensitivity.
5.3 Materials and Methods

5.3.1 Immobilization of antigens on glass surfaces

VN7 and VN6 peptides derived from partial sequences of two individual receptors of the vomeronasal organ [68] were used as an antigen and a control, respectively. Amino acid sequences of the peptides are as follows: FHSRTKFKDGSIFYC for VN7, and VRRISTLYGVVDKQAIC for VN6. Both peptides were synthesized by Takara Shuzo Co., Ltd., Shiga, Japan. For force measurements, aminosilanized glass slides (MAS-coated glass, Matsunami Glass Ind., Ltd., Osaka, Japan) were activated with 0.1 mM of sulfo-LC-SPDP (Pierce, Rockford, IL) in water for one hour, and then washed. VN7 or VN6 peptides were dissolved in dimethyl sulfoxide, diluted to a concentration of 0.5 mM with phosphate-buffered saline (PBS), and mounted on the activated glass for one hour. After the reactions, 5 mM of cysteine in PBS was mounted on the glass and incubated for 30 min to block the remainder of the non-reacted crosslinkers.

To dilute antigens (VN7 peptide) on a glass surface to suitable concentrations, control peptide (VN6 peptide) was mixed with antigen solutions. For example, to dilute VN7 peptide by one hundred times, 99 times molar excess of VN6 was added to the solution of VN7 with a final concentration of 0.5 mM.
5.3.2 Quantification of numbers of antigens immobilized on glass surfaces

For the quantification of the number of crosslinkers which reacted, the optical absorbance of pyridine-2-thione, which is a product of the reaction of sulfo-LC-SPDP with a thiol group, was monitored at a wavelength of 343 nm (molar extinction coefficient is $8.08 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The sulfo-LC-SPDP solutions were mounted on the glass in different conditions, and then the glass surface was rinsed with an excess PBS. After the immobilization of sulfo-LC-SPDP on the aminosilanized glass, 1mM dithiothreitol (DTT, Pierce) was applied to the glass for one hour. The solution was collected and its optical absorbance was measured. The number of sulfo-LC-SPDP immobilized on the glass surface was then estimated by calculating the number of pyridine-2-thione molecules from the optical absorbance, and was normalized with respect to the area of the glass surface. The relationship between the concentrations of sulfo-LC-SPDP solution mounted to the glass and the optical absorbance of pyridine-2-thione was obtained.

5.3.3 Preparation of the AFM tip

To increase the contact area between the sample surface and the AFM tip, we attached a carboxylated polystyrene microbead (Polybead Carboxylate Microsphere, r=5 µm nominally,
Polyscience, Inc., Warrington, PA) to an AFM cantilever (NP, Digital Instruments, Santa Barbara, CA) with epoxy resin using micro manipulators (MMN-1, MMO-202ND, and MN-153, Narisige Co., Tokyo, Japan). Details are given in 2.3.1.

Anti-VN7 antibodies (0.1 mg/mL), which were obtained by following the same procedure of our previous work [68], were covalently crosslinked to microbeads as described below. The carboxylated surface of the microbeads was reacted with 10 mg/mL 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce) and 10 mg/mL N-hydroxysulfosuccinimide (Pierce) in pH 6.0 PBS for 15 min. After a wash with pH 6.0 PBS, the beads were reacted with anti-VN7 antibodies in pH 7.4 PBS for 2 hrs and then washed with pH 7.4 PBS. After the reaction, 20 mM of glycine in pH 7.4 PBS was applied to the beads and incubated for 30 min to block the remainder of the non-reacted parts.

5.3.4 Force measurement and data analysis

Force measurement experiments were carried out on an atomic force microscope (NVB-100, Olympus, Inc., Tokyo, Japan) at room temperature. Force measurements between several concentrations of VN7 antigens and anti-VN7 antibodies were performed in PBS solution in the presence or absence of 50 µM of VN7 peptides. The force mapping mode with a 250 nm Z scan size and 125 nm/sec Z scan rate was used to change the places of measurements on surfaces one
by one. The 16 x 16 µm² scan area was subdivided into 16 x 16 points and measurements were performed at all of the subdivided points.

The force mapping data were transformed to force-extension curves for individual subsections, and "separation work", $W$, was obtained and used as a parameter for comparison of the relative propensity for interaction (see 2.4.1). The contact area $S$ was also calculated from the shape of the force curve according to the Hertzian contact model for a spherical indentor [43-48]. The depth of indentation, $d$, was calculated from the force curve as the difference of piezo displacement and the deflection of cantilever after the probe touched the sample surface [20]. Contact area, $S$, was calculated from the maximum value of $d$ at the end of indentation, according to following formulae.

$$S = 2\pi R^2 (1 - \cos \theta) \quad (5-1)$$

where $\theta$ is defined through

$$\sin \theta = \left(\frac{d}{R}\right)^{1/2} \quad (5-2)$$

with the tip radius, $R$ (see 2.5.1).
5.4 Results and Discussion

5.4.1 Quantification of number of antigens on a glass surface

To quantify the number of antigens on a glass surface, the optical absorbance of pyridine-2-thione, which is a product of the reaction of sulfo-LC-SPDP with a thiol group, was monitored at a wavelength of 343 nm (figure 5.1). Figure 5.1 (B) shows the dependence of the number density of immobilized sulfo-LC-SPDP on the concentration of applied sulfo-LC-SPDP solution. The number density seems to be saturated at about $3 \times 10^6$ per square micrometer. This is a maximum number of peptides immobilized on a surface, and the number density of antigen molecules was likely to be lower than that of sulfo-LC-SPDP because of steric hindrance between antigen molecules. It is difficult to estimate an actual number density of peptides, however, the number density approaches to the maximum number when enough antigen molecules are mounted on the glass, we used the value of maximum number density here after. We chose a 0.1 mM solution of sulfo-LC-SPDP, which corresponds to the number density of about $10^6$ immobilized crosslinkers per square micrometer, and VN7 peptides were immobilized to the crosslinkers. Measurements of separation work, $W$, between VN7 peptides which were immobilized on a glass surface and anti-VN7 antibodies which were immobilized on a microbead were then performed using an AFM.
Figure 5.1
(A) Schematic diagram of reactions of sulfo-LC-SPDP and release of pyridine-2-thione. (B) Dependence of the number density of immobilized sulfo-LC-SPDP on a glass surface on the concentration of applied sulfo-LC-SPDP solution. The fitting curve in exponential is \( y = 2.84 - 2.47\exp(-2.56x) \), \( \chi^2 = 0.3908 \).
5.4.2 Resolution of colloidal probe method to distinguish different number densities of antigens

In order to confirm that $W$ is due to specific interaction between the VN7 antigen and the antibody, measurements of the $W$ between VN6 peptides and anti-VN7 antibodies were also carried out in a control experiment (figure 5.2). In comparison with the measurements obtained using glass surfaces which were coated with VN7 peptides, the $W$ between VN6 peptides and anti-VN7 antibodies was significantly small. Interactions between VN7 peptides and the antibodies were also greatly inhibited after the addition of free VN7 peptide at a concentration of 50 µM to the solution (figure 5.2). These results indicated that the significantly larger $W$ than
that of control experiments was due to specific interactions between VN7 peptides and the antibodies.

In order to estimate the resolution of our method in which a microbead was used as a probe, concentration of VN7 peptide immobilized on glass surfaces was sequentially diluted by mixing suitable amounts of VN6 peptide. The AFM measurements were then made and compared for different number densities of VN7 peptide (figure 5.3). Since the number density of crosslinkers was fixed at about $10^6$ per micrometer square for all conditions, the number density of peptides was estimated as in the order of $10^6$ molecules per micrometer square taking the volume of peptides into account. As shown in figure 5.3, one hundred fold difference in peptide density is easily distinguishable, i.e. between $10 \, \mu m^{-2}$ and $10^2 \, \mu m^{-2}$, between $10^2 \, \mu m^{-2}$ and $10^4 \, \mu m^{-2}$ and between $10^3 \, \mu m^{-2}$ and $10^6 \, \mu m^{-2}$. For ten fold differences, histograms of $W$ are also distinguishable. When the number density is low, for example, histograms obtained for $10 \, \mu m^{-2}$ and $10^2 \, \mu m^{-2}$ are difficult to be distinguished, because of the limitation of contact area (see also figure 5.4). As the number density of antigens was increased, $W$ became gradually larger. There was a clear difference between $10^4 \, \mu m^{-2}$ and $10^5 \, \mu m^{-2}$, only the latter showing a maximum in histogram and could be fitted to a Gaussian curve (figure 5.3 (C)). These results indicate that our method can distinguish ten-fold difference in sample concentrations, such as those of specific receptors on a cell surface. One advantage of our method is that it can simultaneously be applied to many kinds of target molecules such as receptors on a cell surface by changing the kinds of
Figure 5.3

(A) Comparison of $W$ between VN7 antigen and anti-VN7 antibody with varying concentrations of VN7 peptides immobilized on glass surfaces. The concentrations of antigens were diluted by mixing suitable amounts of VN6 peptide. (B) Magnification of Figure 3 (A) with the value of $W$ less than $2.5 \times 10^{-18}$ J. (C) Results of curve fitting for the results at $10^4 \mu m^{-2}$ and $10^5 \mu m^{-2}$ antigen densities. There was a clear difference between $10^4 \mu m^{-2}$ and $10^5 \mu m^{-2}$, in that only the latter showed a maximum in histogram and could be fitted to a Gaussian curve.
probe molecules which are immobilized on the bead without any treatment of sample cells. The work would be of greater value if the antigens could be immobilized on soft material such as the cell surface. The viscoelasticity of the cell surface will have effects on parameters such as contact area, and hence influence the measurements. To reduce the contact area on a cell surface, compression-free force spectroscopy may be useful [69, 70].

5.4.3 Minimum number density at which the antigen could be detected

In order to estimate the minimum number density at which the antigen could be detected using the AFM, probability of detection for $W$ larger than $0.5 \times 10^{-18}$ J was compared as shown in figure 5.4. Weak interactions less than $0.5 \times 10^{-18}$ J that are comparable to nonspecific interactions observable in control experiments were neglected. There is a clear difference in the number density of antigens between less than $10^2 \, \mu m^2$ and more than $10^3 \, \mu m^2$. The mean value of contact area with S.D. was $3.5 \pm 3.1 \times 10^{-2} \, \mu m^2$ ($n = 81$), the large S.D. value was due to small contact area. For the case of $10^3 \, \mu m^2$, 4 to 66 antigen molecules could exist under the contact area, indicating that our method would be able to give an order of estimate about number density of target molecules per contact area, which means quite a high sensitivity. In our method, a microbead was used as a probe to observe distributions of receptors on a cell surface, and the bead diameter was over 100 times larger than usual AFM tips. It is difficult to detect single mol-
Figure 5.4
Probability of detection of W with a value over $0.5 \times 10^{-18}$ J.

ecular interactions on a cell surface using such a large bead, however, our method is useful for the
purpose of receptor mapping. To measure single molecular interactions on a cell surface, use of
conventional AFM tips [49, 71] or reduction of the contact area by compression-free force
spectroscopy [69, 70] may be preferable.