

Dynamic Measurement Method for Bio-molecular Interactions by Using Centrifugal Force

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Measurement of single molecule interaction is performed by various micromanipulation techniques. However, a complex operation is required to perform the measurement, so the measurement throughput is low and a large amount of time is required for statistical analysis. In this paper, we propose a method for measuring intermolecular interactions that applies a tensile force to the intermolecular bond by using centrifugal force and can easily measure binding force. The binding force of an antigen-antibody reaction system was measured by the use of the proposed method as a proof of concept. The measured binding forces were distributed in the range of 19 – 133 pN. The values obtained by use of the proposed method were consistent with the conventional method. This result is promising for applications that require further development, such as the development of a biosensor as a novel measuring technique that is based on the intermolecular interaction measurement.

Keywords Biomolecular interactions, centrifugal microfluidic device, single-molecule measurement

(Received April 18, 2019; Accepted June 19, 2019; Published October 10, 2019)

Introduction

The dynamic measurement of biomolecules by single-molecular force spectroscopy is useful not only for elucidation of physiological functions but also for quantitative evaluation of changes in the physical properties of biomolecules caused by specific diseases, such as cancer and malaria.¹ In recent years, apparatus and technologies have been created to measure the force of sub pN in various ways brought about by the advancement of molecular biology and nanotechnology. Typical single-molecular force spectroscopy methods include atomic force microscopy (AFM),^{2–6} optical tweezers,^{7–11} and magnetic tweezers.^{12–16} Among these single-molecular force spectroscopy methods, AFM is the most widely used method. In this method a ligand-immobilized cantilever is attached to the receptor's immobilized substrate to form a bond for measurement. Then, by pulling the cantilever up until the bond breaks, the binding force can be estimated from the deflection of the cantilever.¹⁷ In the case of AFM, it is necessary to operate the cantilever every time for each measurement, and it takes a large amount of time to statistically analyze the interaction. When considering wider applications of single-molecule interaction analysis, including the diagnostic application of clinical samples, AFM is not suitable due to the time it takes to perform measurements. The optical tweezers method is able to measure molecular interactions by capturing and manipulating microbeads that are immobilized with biomolecules. In the optical tweezers method, it is possible to apply a force of several hundred piconewtons without touching the object being inspected, but as with the

AFM method, the statistical analysis takes too much time for practical applications. Furthermore, photo damage of the target molecule is an issue.¹⁷ In the magnetic tweezers method, the force required to break the bond is measured by pulling magnetic beads that are bound to the substrate by a magnetic force. Using this method it is possible to perform high-throughput interaction measurements by simultaneously applying a magnetic force to a large number of magnetic beads. However, the force applied to the beads differs greatly depending on the relative position of the beads and magnet, which is difficult to control.¹⁷ In addition, as the apparatus of all these methods are large and complicated, they require skilled professionals to perform the measurements. Therefore, we focused on the method using centrifugal force that can apply uniform force,¹⁸ and propose a method that makes it possible to measure interactions for multiple biomolecules more easily than the existing techniques for measuring biomolecule interactions.

Figure 1 shows the measurement principle that we propose. As shown in Fig. 1(a), biomolecules, such as antibodies, are irreversibly immobilized on the surface of two microbeads that have different densities. These biomolecules are reacted with a molecule, such as an antigen, to form a sandwich bead complex. The prepared bead is suspended in a solvent that has a density intermediate between the densities of the two beads, and centrifugal force is applied by rotating it. The combined effects of buoyancy and centrifugal force orientate the beads according to their density (Fig. 1(b)). The bead complex orientates in the direction of the centrifugal force and touches the wall surface of the chamber. Then, the sedimentation force in the direction of the centrifugal force is canceled out by the reaction force, and buoyancy affects the bond of the biomolecules as a tensile force (Fig. 1(c)). As the rotational speed increases, the tensile force also increases, and when the tensile force exceeds the force of

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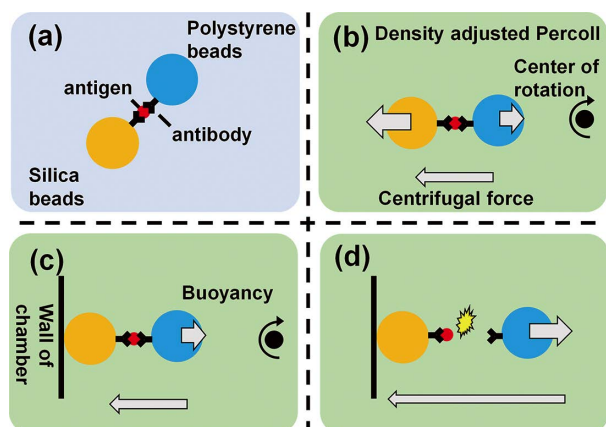


Fig. 1 Schematic of the proposed measurement principle: (a) initial state, (b) settling of the bead complex due to centrifugal force, (c) the force in the direction of gravity is canceled by the counteraction and (d) as the rotational speed increases, the buoyancy also increases and the bond between the beads breaks.

the binding force, the bead complex breaks apart (Fig. 1(d)). Therefore, the binding force between the biomolecules can be estimated from the buoyancy of the low-density bead. When using this method, because it is possible to simultaneously measure interactions among a plurality of biomolecules, statistical analysis can be easily performed. In addition, conventional single-molecular force measurement, which involves directly immobilizing ligands and receptor molecules on a substrate or probe, aimed to obtain basic knowledge on specific interactions between molecules. On the other hand, in this method, the target substance dissolved in a liquid sample can be analyzed by sandwiching the molecule with the microbeads for which the bio-molecules are immobilized in advance. Therefore, it becomes possible to measure the interaction of the non-immobilized molecules in a liquid sample, and applications such as clinical diagnostics can be expected. In this paper, we report on the first proof of concept of bio-molecular interaction measurements based on the proposed measurement principle.

Materials and Methods

Immobilization of antibodies onto beads

Polystyrene (PS) beads (polybead polystyrene microspheres (2.5% solids-latex), 20.0 μm , Polyscience #18329) were used as the low-density microbeads and silica (Si) beads (Sicastar[®], COOH, 20 μm (50 mg/mL), Micromod Partikeltechnologie GmbH #43-02-204) were used as the high-density microbeads. Antibody molecules were immobilized according to the procedure of the PolyLink Protein Coupling Kit for COOH Microparticles (PolyScience, #24350) to covalently fix the beads and antibodies. An outline of the procedure for antibody immobilization onto the beads is described below. First, 12.5 mg of PS beads and Si beads were added to each 2.0 mL tube, then centrifuged at 1000g for 10 min and the supernatant was removed. Then, 0.17 mL of PolyLink coupling buffer included in the kit and 0.2 mL of goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody were added to each tube and the beads were suspended using vortex. Next, 1–2 mg of PolyLink EDAC was added to a new 2.0-mL tube and the beads were mixed together with the solvent. The antibody molecules

were immobilized onto the surface of the beads by stirring the solution overnight at room temperature using a mix rotor.

Reagent preparation and immune reaction procedure

Dulbecco's Phosphate-Buffered Saline (DPBS) was prepared to 100 mM, pH 7.2 to 7.4 and diluted 10 times. Bovine serum albumin (BSA; Albumin from bovine serum, Sigma-Aldrich Japan Co., LLC, Japan, #A 7030-50 G) was dissolved and used at 1% (w/w) in DPBS. After adjusting the specific density by mixing Percoll (GE Healthcare #17-0891-01) and DPBS at a ratio of 7.4:1.6 (v/v) to create a solvent that had a density intermediate between the two types of beads, Tween 20 (Polyoxyethylene (20) Sorbitan Monolaurate, Wako Pure Chemical Industries, Ltd., Japan, #167-11515) was added at 0.05% (v/v). The measured density of this solvent was 1.0943 g/cm³. Tween 20 was also dissolved in DPBS at 0.05% (v/v) and used as a washing solution. For the antigen to be added when preparing the bead complex, the concentration was adjusted to 570 $\mu\text{g/mL}$ by diluting it using Pierce[™] Ms IgG Whole Molecule Control (Thermo, #31903) 10 times with DPBS supplemented with 1% BSA (v/v).

In order to confirm the formation of a bead complex, an observation was carried out as follows. First, PS and Si beads, onto which the antibody molecules were immobilized, were adjusted to 5000 beads/ μL . Then, 6 μL of antibody-immobilized PS beads were added into a 2-mL tube. Next, 2 μL of antigen molecule solution were adjusted by adding 5 $\mu\text{g/mL}$ of antigenic molecule-free DPBS with 1% BSA. The solution was mixed gently with vortex and left at room temperature for 30 min to react. Next, it was centrifuged at 1000g for 1 min. The supernatant was then removed and 1 mL of washing solution added. This washing operation was repeated three times to separate unreacted antigen molecules. After the washing solution was removed, 6 μL of Si beads were added and the solution was mixed gently with vortex. It was then allowed to stand at room temperature for 30 min to react the Si beads with the antigen of the PS beads in order to bind the two beads by an antigen-antibody reaction. Unbound PS beads were separated by suspending the beads by adding 350 μL of Percoll, whose density had been adjusted.

Observation method

A microchip made of PDMS (SILPOT 184, Dow Corning Toray Co., Ltd., Japan) into which a micro-chamber was patterned for observation of bead behavior was prepared by a general soft lithography method.¹⁹ The silicon wafer was coated with a negative photoresist (SU-8 3050, Nippon Kayaku Co., Ltd., Japan) by spin coating to a thickness of 100 μm and baking on a 95°C hotplate for 45 min. After being covered with a photomask and exposed to UV light for 45 s, post baking was performed on a 65°C hotplate for 5 min and on a 95°C hotplate for 15 min. The relief pattern for the micro-chamber was developed with SU-8 developer. Liquid PDMS was prepared with PDMS monomer and a crosslinking agent at a ratio of 10:1 (w/w). Then, it was poured into the mold and the PDMS was cured at 100°C for 1 h. The PDMS was peeled from the mold and the PDMS chip was cut into a 5-mm square. The PDMS chip with the patterned chamber was cleaned ultrasonically with ethanol and water separately for 3 min each. It was then vacuum dried for 25 min and hydrophilized by exposing it to air plasma for 1 min. The hydrophilized PDMS chip was attached to a compact disk-like substrate by self-adhesion with the chamber facing upward (see Fig. 2). To the prepared device, the bead complex prepared in the section above was added dropwise together with the solvent, covered with a 5-mm square glass

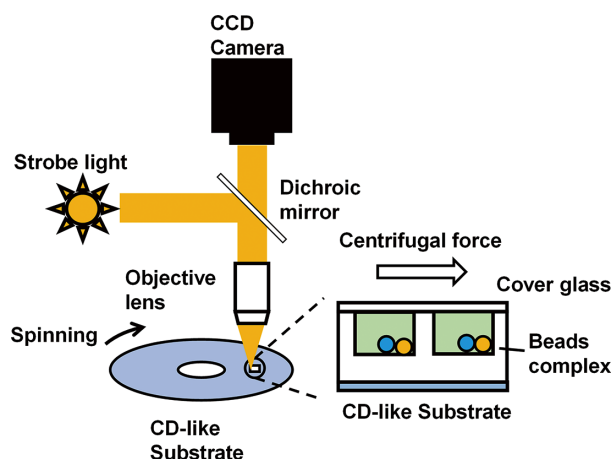


Fig. 2 Schematic of the centrifugal observation apparatus.

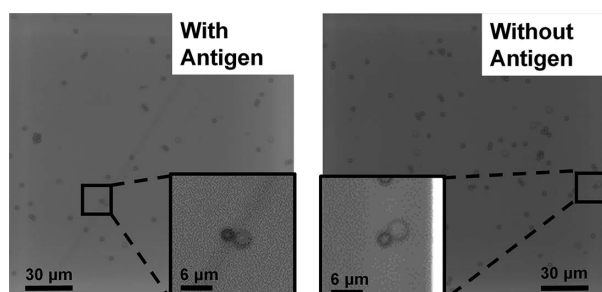


Fig. 3 Photograph of observed microbeads.

cover (MATSUNAMI GLASS Ind., Ltd., Japan), and joined by PDMS self-adhesion to enclose the beads. To apply centrifugal force, a centrifugal observation apparatus developed in the laboratory was used.²⁰ Figure 2 shows an overview of the centrifugal observation apparatus. The prepared device was mounted on the apparatus and rotated. Then, a signal synchronized with the rotation cycle was generated by the encoder. A stroboscope synchronized with the motor emitted a strobe light at the same point in the rotation cycle so that a microscopic image could be gathered.

Results and Discussion

Confirmation of immune complex formation

In order to confirm that the prepared bead complex was formed by an antigen-antibody reaction, observation of the bead complex was carried out for cases with and without the addition of antigen molecules. As shown in Fig. 3, PS and Si beads came in contact with each other, as if they were forming a complex, in both cases with and without the addition of antigen. Therefore, after the removal of unbound PS beads through the addition of high-density Percoll, an observation was carried out to determine the ratio of PS beads bound to Si beads. As shown in Fig. 4, the numbers of observed Si beads with antigen and without antigen before separation were 994 and 1480, respectively. Among them, the numbers of Si beads having a contact point with PS beads were 36 and 40, respectively. On the other hand, the numbers of Si beads counted after separation were 577 and 563, respectively. Among them, the numbers of

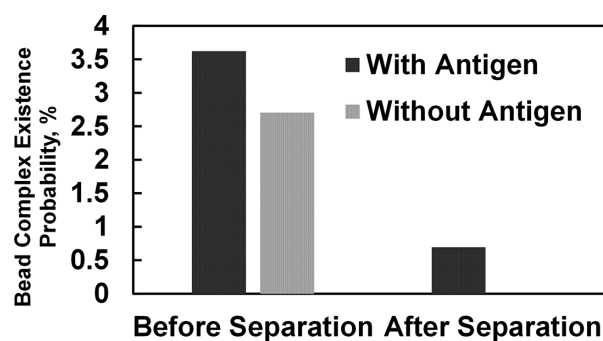


Fig. 4 Measured bead complex ratio.

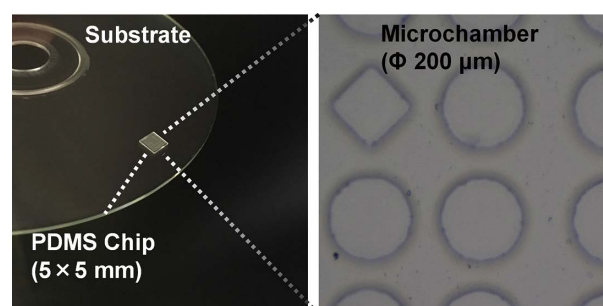


Fig. 5 Photographs of the centrifugal disk and PDMS microchamber.

Si beads having a contact point with PS beads were 4 and 0 respectively. It was confirmed that Si beads bound to the PS beads only when the antigen was added. Using the previously described method, unbound PS beads could be separated and removed, and it was confirmed that the bead complex formed in a series of experiments was bound by an antigen-antibody reaction.

Evaluation of intermolecular interaction

Figure 5 shows an outline drawing of the device used in the experiment. A PDMS chip with several micro-chambers each with a diameter of 200 μm was attached on a CD-shaped substrate. After enclosing the suspended bead complex in the micro-chamber, the behavior of the beads during tensile testing was observed. The spinning of the substrate was controlled to reach a rotational speed of up to 2000 rpm at an acceleration of 50 rpm/s followed by steady rotation for 20 s. Figure 6 shows a snapshot of bond breaking behavior observed during an experiment (Case A). At low rotational speeds up to approximately 13 G the settling force along with the direction of the centrifugal force affected the Si beads and the complex settled down. The Si beads of the complex contacted and were supported by the wall of the chamber and co-enclosed Si beads in the chamber. The force in the direction of centrifugal force was canceled out by counteraction. As the rotational speed increased, the buoyancy acting on the PS beads increased, and it could be seen that the buoyancy was pulled in the direction opposite to the centrifugal force. At 24.5 s after the start of rotation (at this time the rotational speed was 1223 rpm), it was confirmed that the two beads were separated, suggesting the bond between the antigen and antibody was broken.

The centrifugal acceleration acting on the bead and the buoyancy generated by the centrifugal force are calculated as

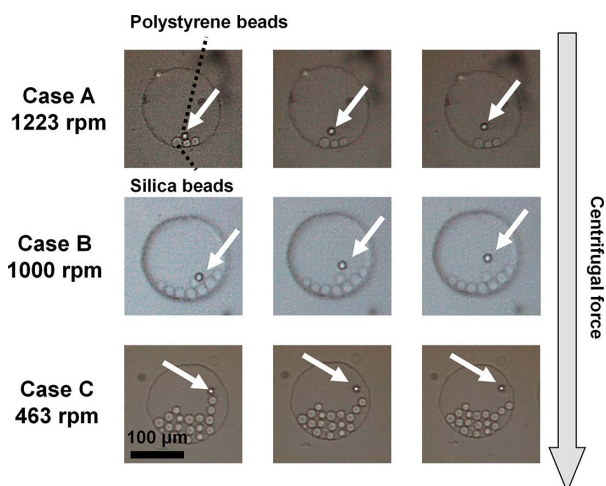


Fig. 6 Observations of three cases of bond breaking behavior.

follows. The acceleration, a , generated due to rotation of the system and angular velocity, ω , are

$$a = r\omega^2 \quad (r: \text{rotation radius [cm]}),$$

$$\omega = 2\pi N/60 \quad (N: \text{number of rotations [rpm]}).$$

Therefore, the relative centrifugal force (RCF) applied to the rotating beads can be estimated by

$$\text{RCF} = a/g \quad (g: \text{gravitational acceleration}).$$

When the fluid density is ρ_f , the buoyancy, F_b , acting on an object of volume V is

$$F_b = \rho_f V g [\text{N}].$$

When the density of the object is ρ_s , the resultant force F of buoyancy and gravity is

$$F = (\rho_f - \rho_s) V g [\text{N}].$$

To determine the tensile force acting on the bead due to centrifugal force, the gravitational acceleration, g , in the above equation is replaced with RCF.

Figure 6 shows a snapshot of the behavior of the beads in three cases (cases A, B, and C) that were successfully observed. Using the above formula, the buoyancy acting on the PS beads when the beads were separated could be estimated to be approximately 133, 86, and 19 pN for cases A, B, and C, respectively. It has been reported by another study, which used AFM, that the binding force between biotin and anti-biotin antibodies was estimated to be 111.5 ± 98.6 pN.²¹ It can therefore be concluded that the obtained results are consistent with the results of AFM measurement, because the same order of magnitude of binding force was estimated. Therefore, we have succeeded in demonstrating the proposed biomolecular interaction measurement principle.

Discussion

The measured binding force between the antigen and the antibody are shown in Fig. 7. The estimated binding forces are distributed between 19–133 pN. As a result of studies using

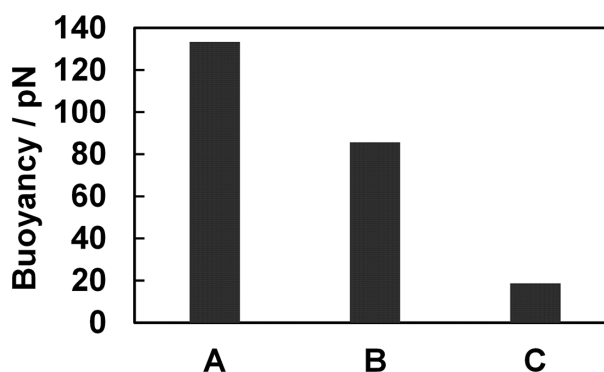


Fig. 7 Calculated antigen-antibody binding forces.

AFM, the binding force of antigen-antibody reaction is several tens to one hundred and several tens pN, so it is considered that is the similar value in comparison with existing measurement techniques.^{21–23} The binding forces measured in our experiment have a large variation. One factor that may contribute to this is the very large curvature radius of the microbeads, when compared to the size of the antibody molecules. Assuming the size of an antibody molecule is approximately 5 nm, when the distance between the bead surfaces is less than 10 nm, an antigen-antibody bond can be formed. Assuming two ideal spherical beads come into contact, the contact area is estimated to be a circle of $0.30 \mu\text{m}^2$. Therefore, when the antibody molecules are immobilized on beads to a high density, it is possible that multiple antibody molecules are involved in the bond between the beads.

It was also considered that buoyancy does not act on the binding molecules in an exactly vertical manner, because the bead complex was sometimes tilted. In case B, the bead complex seems tilted at an angle of approximately 45° . In this case, because the tensile force applied to the bond between the molecules is approximately 0.7 times in the case with vertical orientation, it is considered that the rotational speed required for separation and the estimated binding force will become larger. Also, at the same time, 0.7 times the buoyancy acts as a shearing force against the bond between the molecules. As the bond shear force is unknown, a study that examines and evaluates the shear force is considered necessary.

In this experiment, the antibody immobilized onto the beads is a polyclonal antibody. Several types of antibody molecule exist that have binding activity against a single antigenic molecule. Therefore, even when the binding force of a single bond is detected, it is considered that different binding forces can be detected depending on the difference in epitope of the antibody. So, it is considered as a factor that the detected binding force is diversely distributed. Furthermore, in the bead complex of case A, two Si beads appear to be binding to one PS bead. It is considered that this is a combination of two factors: the angle between the PS beads and the Si beads, and the contribution of multiple bonds to the formation of the bead complex. Because the binding force calculated in case A is the highest, it is considered that the above factors contribute to variations of the measured binding force. Therefore, it is necessary to develop a bead complex formation process in which PS beads and Si beads form a complex at a ratio of 1:1.

It was considered that the binding power between antigen and antibody estimated in this experiment was distributed due to factors such as multiple antibody molecules involved in the formation of the bead complex. In order to evaluate the effect

of multiple binding, it is necessary to quantify the density of antibody immobilization on beads. To estimate the density of antibody immobilized on beads, it is necessary to evaluate the amount of antibody molecules immobilized on beads. Therefore, it is necessary to perform a quantitative evaluation of the density of immobilization of antibody molecules on microbeads.

Conclusions

In this paper, we devised a method to measure the interaction between biomolecules by applying a tensile force between two particles by using centrifugal force. In addition, we successfully demonstrated the measurement principle by conducting experiments using mouse IgG antigen and anti-mouse IgG antibody as samples. The greatest advantages of the proposed measurement principle are that it is capable of applying force uniformly to several molecules and easily performing parallel measurement of binding forces. Information on single-molecule interactions, which require large amounts of time to acquire using conventional technology, can be obtained immediately by parallel measurements. Therefore, we expect the development of new bio-sensing technology utilizing the difference between biomolecular interactions.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science KAKENHI Grant Numbers JP17H04746 and JP15K12505.

Supporting Information

We provide supplementary information on the measurement of the density of polystyrene beads used in the calculation of intermolecular interactions. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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