Detecting CD20-Rituximab specific interactions on lymphoma cells using atomic force microscopy

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Elucidating the underlying mechanisms of cell physiology is currently an important research topic in life sciences. Atomic force microscopy methods can be used to investigate these molecular mechanisms. In this study, single-molecule force spectroscopy was used to explore the specific recognition between the CD20 antigen and anti-CD20 antibody Rituximab on B lymphoma cells under near-physiological conditions. The CD20-Rituximab specific binding force was measured through tip functionalization. Distribution of CD20 on the B lymphoma cells was visualized three-dimensionally. In addition, the relationship between the intramolecular force and the molecular extension of the CD20-Rituximab complex was analyzed under an external force. These results facilitate further investigation of the mechanism of Rituximab’s anti-cancer effect.

atomic force microscopy, single-molecule force spectroscopy, CD20 antigen, Rituximab


Virtually all knowledge of cellular chemical processes has been deduced from in vitro measurements [1,2], and reflects an average of results from multiple molecules and many cells [3]. Undoubtedly these results correctly depict the behavior of multiple molecules, and provide information on cellular chemical processes [4]. However, these types of measurements can mask rare events and the properties of individual molecules [5]. Mounting experimental evidence indicates that these measurements may not correctly reflect the in vivo situation. By contrast, single-molecule experiments could be used to study the underlying molecular mechanisms of single biologically important molecules [6]. Analysis of single molecules would provide insight into cellular physiological.

There are approximately 20 single-molecule techniques available, including fluorescence and force-measuring techniques. Among these, atomic force microscopy (AFM) is the only tool that can be used to detect, localize and manipulate single molecules on cells [5]. Unlike other techniques, it can be applied to fluids, which makes observation of the dynamic physiology of living cells possible [7]. Additionally AFM has nanometer resolution [8], is easy to control, causes little damage to samples with small scan force [9], and the sample preparation is simple and fast [10]. Consequently, AFM has been widely applied in the investigation of cell physiology, and has provided new insights
into biological activities at the cellular and molecular levels [11]. AFM methods can be used to investigate molecular mechanisms of critical diseases [12], and have positively impacted the search for new biomarkers [13] and drug discovery [14].

Single-molecule force spectroscopy (SMFS) [15] can be used to detect receptor-ligand specific recognition. In this method the ligands are bound to the AFM tip and the receptors are bound to the support surface. SMFS was first applied in the mid 1990s to the measurement of binding forces for biotin-avidin [16] and human serum albumin (HSA) to anti-HSA [17]. Subsequently it was applied to various types of molecular specific binding. Currently, SMFS is applied to many areas ranging from detecting the specific recognition of receptors immobilized onto the substrate [18] to directly detect the specific recognition of receptors on the cell surface [11,12,19–22]. The detection of the receptor-ligand specific recognition on the cell surface by SMFS aids understanding of the molecular dynamics of receptor-ligand complexes during the association and dissociation processes [8] and the distribution of receptors on the cell surface [15,22,23].

Clinical application of Rituximab over the past decade has indicated that the responses of different subtypes of lymphomas are markedly different. To investigate these differences, AFM methods can be used to explore the molecular mechanisms of Rituximab. In this work, AFM was used to study the CD20-Rituximab specific recognition under near-physiological conditions. Methods for measuring the molecular force by AFM tip functionalization, and visualizing the CD20 distribution on cell surface are presented. These methods will facilitate future investigations of the underlying molecular mechanisms of the efficacy of targeted therapies for critical diseases such as cancer.

1 CD20 and Rituximab

Non-Hodgkin’s lymphoma is a common malignant tumor that constitutes 4%–5% of all tumors [24]. Approximately 85% of non-Hodgkin’s lymphomas in adults are of B cell origin [25]. The approval of the Rituximab by the US Food and Drug Administration in 1997 revolutionized the treatment of B cell lymphomas. Rituximab induces depletion of the target cell by binding with the CD20 antigen on the cell surface. The use of Rituximab, particularly in combination with various chemotherapy/radiotherapy regimes, has significantly improved all aspects of the survival statistics for lymphoma patients [26]. Consequently, it has become the mainstream treatment for B cell lymphomas.

The CD20 antigen belongs to the cluster of differentiation (CD) family of leukocyte differentiation antigens. It is a tetra-spanning membrane protein and has 297 amino acids with both the amino and carboxy termini of the protein located inside the plasma membrane [27]. In addition to the transmembrane form, various soluble forms of CD20 have been detected circulating in the serum of patients with chronic lymphocytic leukemia [28]. CD20 is highly expressed on >90% of B cell lymphomas and rarely on stem cells and plasma cells [29]. In combination with its rare internalization or shedding [28], this makes CD20 an ideal target for the treatment of B cell lymphomas [30].

Rituximab is a chimeric monoclonal antibody (mAb) composed of murine variable regions and human IgG1 constant regions [31]. In vitro experimental evidence indicates that Rituximab acts by three main mechanisms to deplete the target cell after CD20-Rituximab binding (Figure 1): first, by antibody dependent cellular cytotoxicity (ADCC), where Rituximab binds to the Fc gamma receptors of natural killer (NK) cells, which triggers the release of cytotoxin to kill the targeted cell [32]; second, by complement dependent cytotoxicity (CDC), where Rituximab activates the complement system and generates the membrane attack complex (MAC) on the targeted cell, which induces lysis of the targeted cell [33]; third, by programmed cell death (PCD), where Rituximab induces apoptosis of the targeted cell [28]. As the first generation of mAb, the success of Rituximab in clinical application has boosted the development of the anti-CD20 mAb. The second generation mAb is humanized to reduce immunogenicity, and the third generation mAb is humanized with an engineered Fc region designed to improve therapeutic performance [26].

2 Materials and methods

2.1 Sample preparation

The cells used in the experiments were from Burkitt’s lymphoma Raji cell line cultured at 37°C (5% CO₂) in RPMI-1640 culture medium containing 10% fetal bovine

![Figure 1](https://via.placeholder.com/150) CD20-Rituximab binding induces lysis of the target cell. A, CD20 antigen structure. B, Structure and mechanism of Rituximab.
serum. To prevent displacement by the scanning probe [34] when obtaining AFM images in a fluid, cells should be tightly immobilized on a flat support [35]. There are two main methods for cell immobilization: poly-L-lysine, and porous polymer membrane [36]. In this study, poly-L-lysine was used to immobilize the suspension of Raji cells onto a glass slide, and glutaraldehyde [37] was used to chemically fix the cells.

2.2 AFM imaging

AFM measurements were performed using a Nanoscope VI Dimension 3100 AFM (Veeco Company) and oxide-sharpened Si$_3$N$_4$ tips (DNP-S, radius 10–40 nm, Veeco Company) with a normal spring constant 0.06 N m$^{-1}$ (Figure 2). The spring constant was calibrated using a Thermal Tune Adapter (Veeco Company). The normal length, width and thickness of the cantilever were 205, 25 and 0.6 μm, respectively. The experiments were performed in PBS at room temperature. The sample was placed into a petri dish full of PBS, and the AFM probe was guided onto the cells with the assistance of a charge-coupled device (CCD) camera (Figure 2B). A large-scale scan was performed first to locate the cells. Then after setting the parameters of the interface operating software (Nanoscope, V6.13), an exact scan was performed. The scan rate was 1 Hz, the scan line was 256, the sampling number was 256, and the scan force was 50 pN.

2.3 Fluorescence microscopy

The distribution of CD20 on the lymphoma cell surface was qualitatively measured using Rituximab and goat anti-human IgG. First, the cell suspension was dropped onto the glass slide covered with poly-L-lysine, and the slide was placed in a petri dish with PBS. Then Rituximab solution was added to the petri dish, and the dish incubated for 1 h. Following incubation the sample was washed with PBS to remove unreacted Rituximab. Fresh PBS and FITC-labeled goat anti-human IgG solution were added to the petri dish, and this was followed by incubation for 1 h. Finally, the sample was washed with PBS and then placed on the stage of a fluorescence microscope. A blue filter was used to obtain fluorescence images of the sample.

2.4 Tip functionalization

The heterobifunctional linker N-hydroxysuccinimide-polyethylene glycol-maleimide (NHS-PEG-MAL) [21,38,39] was used to link Rituximab to the AFM tip. This linker has an NHS ester on one end and an MAL ester on the other (Figure 3). To link together Rituximab and the AFM tip, the tip was coated with amino groups using tip functionalization reagents (Sigma-Aldrich, USA) and following an established procedure [40]. The Rituximab, which was obtained from the Chinese Affiliated Hospital of Military Medical Academy of Sciences, was treated to form thiol groups. The linker was then added and the NHS ester function on the NHS-PEG-MAL formed a stable amide bond with an amino group on the AFM tip. The MAL group reacted with a protein thiol resulting in a disulfide linkage between PEG and the protein [39].

2.5 Measuring the molecular force

The functionalized tip was used to obtain force curves and detect the CD20-Rituximab binding force on the surface of lymphoma cells. When CD20-Rituximab specific binding occurred there was a distinct abrupt peak in the retracting portion of the force curve (Figure 3). Receptor-ligand bind-
ing has a low probability (<30%) [21,41], so often hundreds of force curves (500–1000) [19] are required for one measurement. A blocking experiment [8] was used to demonstrate the specificity of CD20 and Rituximab. In this case the force curves were obtained with the functionalized tip after adding sufficient Rituximab to mask the CD20 binding sites.

There can often be more than one peak in the force curve [21,42] due to the formation of several CD20-Rituximab bonds in one measurement. The Poisson analysis method was developed by Stevens et al. [43] to solve the problem of computing the single receptor-ligand binding force from the force curves obtained by AFM.

\[
\frac{\sigma^2}{\mu} = F.
\]  

(1)

Using the variance (\(\sigma^2\)) and mean (\(\mu\)) from each measurement the single receptor-ligand binding force at this loading rate can be calculated from Equation (1). If several measurements at the same loading rate are performed, we can plot variance against mean and the single receptor-ligand binding force can be calculated from the slope of the straight line.

### 3 Results and discussion

#### 3.1 AFM imaging

AFM is currently the only tool that can be used to obtain high-quality images of live cells and perform force measurements [44]. Although many advances have been made in imaging live cells using AFM, the types of living cells that can be imaged are still limited [45] to microbial cells [7,11] and adherent mammalian cells [19]. Microbial cells have hardy cell walls and high-quality AFM images can be obtained without chemical fixation. Adherent mammalian cells can bind to a support, and for tighter immobilization poly-L-lysine can be used. High-quality AFM images can be obtained of these cells in contact mode with a very small scan force (<100 pN) [19]. Acquiring high-quality AFM images of live suspension mammalian cells is challenging due to their growth in suspension and the softness of the cell surface. The AFM contact images of lymphoma cells fixed by glutaraldehyde are shown in Figure 4.

#### 3.2 Fluorescence microscopy

The distributions of CD20 antigen on fixed lymphoma cells and unfixed lymphoma cells were qualitatively measured with secondary antibody fluorescence labeling, respectively (Figure 5). After being fixed with glutaraldehyde, the whole cells appeared with luminous green fluorescence, while the fluorescence of the unfixed cells was weak and mainly around the cells.

### 3.3 Measuring the molecular force

When the specific binding event occurred, there were one or more distinct abrupt peaks in the retracting portion of the force curve (Figure 6A). For the force curves with more than one peak only the last peak was included in the analysis [42]. After adding the free antibodies solution to bind to the CD20 sites on the cell surface, no distinct abrupt peaks appeared in the retracting curve (data not shown). The normal tip was also used to obtain force curves on the lymphoma cell surface and there were no distinct abrupt peaks (Figure 6B). This demonstrated the specificity of CD20-Rituximab binding. Four measurements, each composed of 100 individual force measurements, were acquired at a loading rate of 1.4 \(\times\) 10^6 pN s\(^{-1}\). The histogram of the binding forces computed from these force curves is shown in Figure 6C. From Gaussian fitting we found the binding force was about 320 pN, and was composed of several CD20-Rituximab pairs. The mean and variance of the four measurements were computed as (mean, variance) 0.2567, 0.0038; 0.3487, 0.0059; 0.6193, 0.041; and 0.8628, 0.0472. With the Poisson analysis method, the dissociation force of a single CD20-Rituximab bond at a loading rate of 1.4 \(\times\) 10^6 pN s\(^{-1}\) was about 79 pN (Figure 6D).
force quantitative experiments with computer-aided engineered antibody design aids understanding of the structural basis of an engineered antibody and the binding functionality to its target antigen in pharmaceutical industries [47].

3.4 CD20 distribution on the cell surface

The distribution of CD20 on the lymphoma cell surface was visualized with the functionalized tip in the lift mode (Figure 7). In this mode, the main trace is first made to typically measure the topography, and then the lift trace is made along the main trace to produce an image of the CD20-Rituximab specific recognition. Because any disturbance from the topography has been eliminated, the lift trace is only influenced by the CD20-Rituximab binding. Consequently, the location of CD20 on the cell surface can be clearly seen. The lift scan height is the distance between the main trace and the lift trace. When the normal tip was used to scan the surface of lymphoma cells to obtain the phase image in the lift mode, there was no distinct abrupt peak in the three-dimensional phase image (Figure 7B). By contrast, when the functionalized tip was used to obtain the phase image, there were many distinct abrupt peaks in the three-dimensional phase image (Figure 7C). These peaks correspond to the distribution of CD20.

Dufrene et al. [22,23] used the gray mapping method to characterize the distribution of molecules on the cell surface. In this method, the functionalized tip is used to obtain an

Molecular forces reflect the interplay of chemical, biological and physical interactions [46]. Using SMFS to investigate molecular forces can provide new insights into molecular biology [18,21]. Recent efforts of single-chain fragment variable (scFv) indicate that the binding force between CD20 and anti-CD20 antibody is closely related to the linker peptide [47]. Combination of single-molecule

Figure 6 Measuring the CD20-Rituximab binding force with SMFS. Force curves obtained with the functionalized tip (A) and the normal tip (B). (C) Histogram of CD20-Rituximab binding force. (D) Computing the binding force with the Poisson analysis method and the slope of the fitting line indicate the unbinding force is 79 pN at a loading rate of $1.4 \times 10^7$ pN s$^{-1}$.

Figure 7 Visualizing the distribution of CD20 on the cell surface with lift mode method. A, Lift mode scan principle. Three-dimensional phase image with the normal tip (B) and with the functionalized tip (C).
array of force curves (16×16) in a local area (400 nm×400 nm). The forces computed from these force curves are mapped into a two-dimensional gray image. The gray map reflects the molecular distribution on cell surface. Compared with the gray mapping method, the lift mode method is more intuitive and the molecular distribution can be visualized as three-dimensional images.

3.5 Stretching the CD20-Rituximab complex

The relationship between the intramolecular force [48] and molecular extension of the CD20-Rituximab complex under an external force was measured (Figure 8). The extension of the CD20-Rituximab complex was computed by subtracting the deflection of the cantilever from the Z movement. The intramolecular force was equal to the product of the cantilever deflection and the elastic constant of the cantilever. The CD20-Rituximab complex appears as a nearly linear spring before its rupture. Research indicates that L- and P-selectin [49] and the Wsc1 protein [11] are like linear springs. During stretching with the external force, the length of the complex increased gradually because the subunits of the protein changed from folded to unfolded, extended state [50]. When the extension reached its maximum length, the CD20-Rituximab complex ruptured and the deflection of the cantilever became 0.

4 Conclusion

AFM can be used to investigate the underlying mechanisms of cellular physiology at the cellular and molecular levels. In this work, AFM SMFS was used to explore the CD20-Rituximab specific binding interaction. The CD20-Rituximab binding force was measured quantitatively, and the distribution of CD20 on the lymphoma cell surface was visualized as three-dimensional phase images. In addition, extension of the CD20-Rituximab complex was analyzed under an external force, and it appeared as a linear spring. These results will facilitate further investigation of the molecular mechanism of the efficacy of Rituximab.

![Figure 8](image_url) CD20-Rituximab complex appears as linear spring under an external stretching force.

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