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Quantifying The Adhesion Forces of Lymphoma Cells by AFM Single-cell Force Spectroscopy*

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Abstract Cell adhesion plays an important role in regulating diverse physiological functions of cells, and quantitatively characterizing the adhesive behaviors at single-cell level benefits understanding the biology of cells. The advent of atomic force microscopy (AFM) provides a powerful method for investigating the biophysical properties of biological systems at micro/nanoscale under aqueous conditions, and particularly AFM-based single-cell force spectroscopy (SCFS) is able to measure the adhesion forces of single cells. Nevertheless, current SCFS assays are commonly performed on adherent cells, and SCFS studies on mammalian suspended cells are still scarce. In this work, AFM-based SCFS was utilized to measure the adhesion forces of lymphoma cells. First, the adhesion forces between lymphoma cells and rituximab (an antibody which binds to the CD20 antigen on lymphoma cells to activate immunotherapy) were investigated. Then the effects of antibody concentration and experimental parameters on the adhesion force measurements were investigated. Next, the intercellular adhesion forces between lymphoma cells were quantified. The research demonstrates the capabilities of AFM-based SCFS in detecting the adhesive behaviors of mammalian suspended cells and also provides novel insights into the adhesion of lymphoma cells, which will have potential impacts on single-cell biomechanical assays.

Key words atomic force microscopy, single-cell force spectroscopy, cellular adhesion, lymphoma cell, rituximab
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Cell adhesion plays an important role in the physiological functions of cells. Cell adhesion is closely related to a wide range of biological processes, including embryonic development, tissue assembly, cellular communication, inflammation and wound healing, tumor metastasis, cell culturing, as well as viral and bacterial infection[1]. The adhesive capability of cells dynamically changes during cellular physiological processes. For example, during tumor metastasis, the cancerous cells need to firstly decrease the cell adhesion to detach from the primary tumor for migration[2]. When the cancerous cells squeeze into blood vessels, the cancerous cells need to increase their cell adhesion to tightly adhere to the blood vessel wall for withstanding the blood flow. Adhesive interactions between cells and their environments trigger signaling pathways that are involved in the fulfillment of cellular biological functions[3]. Deviations of cell adhesion from their normal behaviors, for example, the abnormal of cell adhesion to various biomaterial-based matrices, are often accompanied with the pathological changes inside the cells, which eventually promote the appearance of diseases in living organisms[4-5]. Hence, investigating cell adhesion is of important significance for understanding the mysteries of life activities.

The advent of atomic force microscopy (AFM)

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provides a novel powerful tool for investigating the adhesive behaviors of cells at single-cell and single-molecule levels. Besides AFM, diverse single-cell and single-molecule techniques have been developed for characterizing the forces involved in cellular and molecular interactions, including optical tweezers, magnetic tweezers, and biomembrane force probe[6-8]. For practical reasons AFM is the most widely used method. AFM probes cell adhesion by attaching a cell onto the cantilever of AFM's probe and then using the cell probe to directly sense the adhesive interactions between the cell on the probe and the substrate (the substrate can be biomaterials or cells), which is called single-cell force spectroscopy (SCFS)[9-10]. AFM is able to measure biological forces that span orders of magnitude (μN−pN), which allows AFM to investigate a wide range of molecular interactions ranging from receptor-ligands on cell surface to cell-substrate adhesions in near-physiological conditions[11]. For SCFS, the contact between AFM tip and cell can be controlled precisely and the effect of inhibitors on cell adhesion can be examined directly, which facilitate the studies of cell adhesion. SCFS has been widely used to investigate cell-substrate[12-14] adhesion and cell-cell adhesion[15].

In this work, we used AFM-based SCFS to quantitatively investigate the molecular and cellular adhesive interactions of lymphoma cells. The molecular adhesion between lymphoma cells and rituximab and the cellular adhesion between lymphoma cells were studied. Improving the efficacy of molecular targeted drugs has been a challenge urgently needing to be addressed for providing adequate therapies for cancer patients in the coming era of personalized medicine. Non Hodgkin's lymphoma is a kind of common malignant tumors. About 85% of non Hodgkin's lymphoma is B cell lymphoma[16-17]. In 1997, the U. S. Food and Drug Administration (FDA) approved the monoclonal antibody targeted drug, rituximab, for the treatment of B-cell lymphomas[18]. Rituximab is able to specifically bind to the CD20 antigen on the surface of B lymphoma cells to kill cancerous cells by activating immune attack[19]. Rituximab therapy combined with traditional chemotherapy / radiotherapy significantly improves the survival rates of lymphoma patients[20], which has become the mainstream treatment of B-cell lymphomas. Despite the unprecedented success of rituximab in the treatment of B-cell lymphomas, clinical practice has also shown that there are many B-cell lymphoma patients who are insensitive to rituximab and do not benefit from the rituximab therapy. Therefore, the urgent issue needing to be addressed is developing novel anti-CD20 antibodies which have improved efficacy compared with rituximab[21]. In this case, investigating the force interactions between rituximab and lymphoma cells is of fundamental significance for understanding the actions of rituximab[22]. Here, detailed procedures are presented to quantify the molecular and cellular adhesion forces involved in lymphoma cells with the use of AFM-based SCFS, which will have potential impacts on evaluating drug actions at single-cell level.

1 Materials and methods

1.1 Materials and reagents

The biochemical materials used in this study include acetone, biotin-conjugated BSA (Bioss Antibodies, Beijing, China), PBS (Thermo Scientific, Waltham, MA, USA), Streptavidin (Sigma, Merck KGaA, Darmstadt, Germany), biotin-conjugated concanavalin A (Sigma, Merck KGaA, Darmstadt, Germany), 3-Aminopropy triethoxysilane(Sigma, Merck KGaA, Darmstadt, Germany), 25% glutaraldehyde (Sinopharm Chemical Reagent Co., Ltd. Shanghai, China), Rabbit-anti-human CD20 (Bioss Antibodies, Beijing, China), FITC-conjugated horse-anti-rabbit secondary antibody (Bioss Antibodies, Beijing, China), CFDA SE (Beyotime, Shanghai, China), and poly-L-lysine (Solarbio, Beijing, China). The AFM probe type used here is MLCT-O10 (Bruker, Santa Barbara, CA, USA). Rituximab was provided by the Affiliated Hospital of Military Science Academy of the PLA (Beijing, China).

1.2 Cell culture and preparation

B lymphoma cells were obtained from Burkitt lymphoma Raji cell lines which were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Raji cells were cultured in RPMI 1640 medium (Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum at 37 ℃ (5% CO_2). The 2 ml Raji cell suspension was centrifuged at the speed of 1000 r / min for 10 min. After centrifugation, the supernatant was removed. Then 1 ml PBS was added into the tube to resuspend the Raji cells.

1.3 Functionalization of AFM cantilevers

According to the published protocol[23], the tipless cantilevers with a nominal spring constant of 0.01 N / m were coated with concanavalin A. The tipless cantilevers were firstly cleaned by UV-radiation for 45 min. Then the cantilevers were incubated in 50 μl of biotin-conjugated bovine serum albumin (BSA) (0.5 g/L solution in $NaHCO_3$) at 37 ℃ overnight. After incubation, the cantilevers were washed with PBS for three times to remove the unbound molecules and then incubated in 50 μl of streptavidin (0.5 g / L solution in PBS) for 30 min. After washing the cantilevers with PBS for three times, the cantilevers were incubated in 50 μl of biotin-conjugated concanavalin A (0.4 g/L solution in PBS) for 30 min. After incubation, the cantilevers were washed with PBS for three times.

1.4 Substrate functionalization

Rituximab molecules were coated on the substrate of Petri dishes whose diameter was 60 mm. The substrate of the Petri dish was divided into several areas with the use of a marker pen, which facilitated us to coat each area of the substrate with different concentrations of rituximab. The dish was firstly silanized by 2% APTES for 10 min. After silanization, the dish was rinsed with pure water. Then the dish was treated by 0.5% glutaraldehyde for 30 min. After washing the dish with pure water, the six regions at the substrate of the dish were incubated with different concentrations of rituximab for 30 min. For control, the substrate was incubated with BSA. After incubation, the dish was washed by PBS and then free PBS was added into the substrate to keep the activities of the molecules coated on the substrate of the dish.

In order to examine whether rituximab had been coated on the substrate of Petri dish, fluorescence microscopy experiments were performed. The Petri dish coated with rituximab was firstly incubated with rabbit-anti-human CD20 antibody solution for 30 min at 37 ℃. After incubation, the Petri dish was washed by PBS for three times. Then the substrate was incubated with FITC-conjugated horse-anti-rabbit secondary antibody for 30 min. After incubation, the Petri dish was washed by PBS for three times, and then fluorescence images were recorded. For control, Petri dish without rituximab functionalization was also observed. As shown in Figure 1, no fluorescence was observed for the Petri dish without rituximab functionalization, while the Petri dish with rituximab functionalization exhibited bright fluorescence, indicating that rituximab had been coated on the substrate of the Petri dish.

1.5 Single−cell probe preparation

The single-cell AFM probe was prepared by attaching single cells onto the concanavalin A-coated AFM tipless cantilever based on AFM micromanipulations with the assistance of optical microscopy (Catalyst AFM, Bruker, Santa Barbara, CA, USA), as shown in Figure 2. The Raji cell suspension was added into the sub-area of the substrate of the Petri dish (this sub-area was not coated by rituximab). The cells deposited onto the substrate in a few minutes. Then the concanavalin A-functionalized tipless cantilever was moved to one Raji cell under the guidance of optical microscopy (Figure 2a). Next, the cantilever was controlled to gradually approach and contact the cell for 30 s with a contact force 4 nN (Figure 2b). After that, the cantilever retracted from the substrate and the cell was attached to the AFM cantilever to form single-cell probe (Figure 2c).

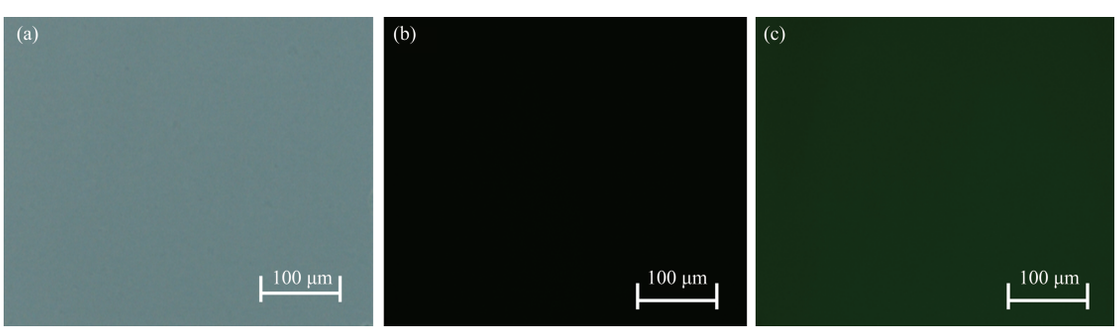

Fig. 1 Fluorescence microscopy experiments verifying substrate functionalization

（a） Optical bright field image of the substrate of the Petri dish. （b） Fluorescence image of the substrate of the Petri dish without rituximab functionalization. （c） Fluorescence image of the substrate of the Petri dish coated with rituximab.

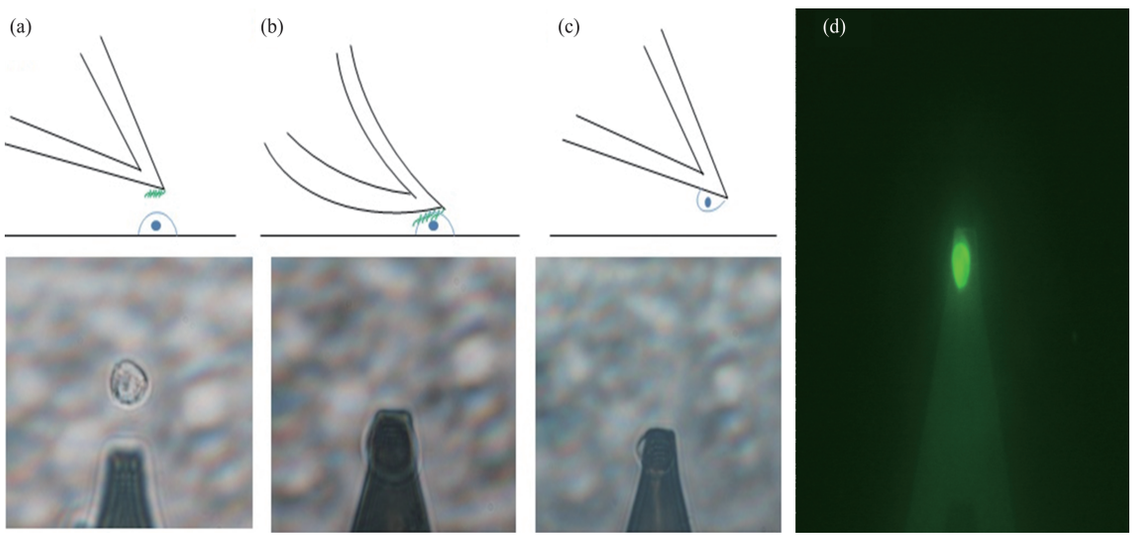

Fig. 2　Preparation and activity verification of single−cell AFM probe

（a-c） Schematic and optical images of preparing single-cell probe. The optical images are under the schematic diagrams. （a） The concanavalin A-coated cantilever was approaching a single Raji cell deposited on the substrate. （b） The cantilever contacted the cell and dwelt for 30 s to allow the binding of cell to the concanavalin A on the cantilever. （c） The cantilever retracted from the substrate and the cell was attached to the cantilever to form single-cell probe. （d） The single-cell probe was stained with CFDA SE and the fluorescence indicated that the cell on the cantilever was alive.

In order to examine the activities of the single-cell probe, the cell attached to the AFM probe was stained with CFDA SE. The AFM single-cell probe was placed in 1 ml CFDA SE working solution and then incubated at 37 ℃ for 15 min. After incubation, the probe was washed by PBS for three times. Figure 2d shows the fluorescence image of the single-cell probe stained by CFDA SE. The cell attached to the cantilever strikingly exhibited green fluorescence, indicating that the cell attached onto the probe was alive.

1.6　Single−cell force spectroscopy

The procedure of using single-cell force spectroscopy to measure the adhesion forces between Raji cells and rituximab-coated substrate was shown in Figure 3. Firstly, the single-cell probe was moved to gradually approach and contact the rituximab-coated substrate with a constant loading force (the loading force is adjusted by changing the trigger threshold of force ramp in the user interface of AFM nanomanipulation software). The single-cell probe dwelt on the substrate for a period of time and then retracted from the substrate. During the retract process, the rupture between cells on the cantilever and rituximab on the substrate resulted in the specific force peaks in the force curve. The magnitude of the force peak corresponded to the cellular detachment force. Figure 3a shows the optical image of measurements and Figure 3b shows the recorded representative force curves. We can clearly see the force peaks in the retract curve, which indicates the CD20-rituximab unbinding events. In some cases, multiple molecular unbinding events were observed from the force curve, which exhibited stepped peaks (top curve in Figure 3b). In some cases, single unbinding events were observed, which exhibited individual peaks (bottom curve in Figure 3b). For each sub-area of the substrate coated with different concentrations of rituximab, 100 force curves were recorded. For control, the substrate coated with BSA was also used for measurements.

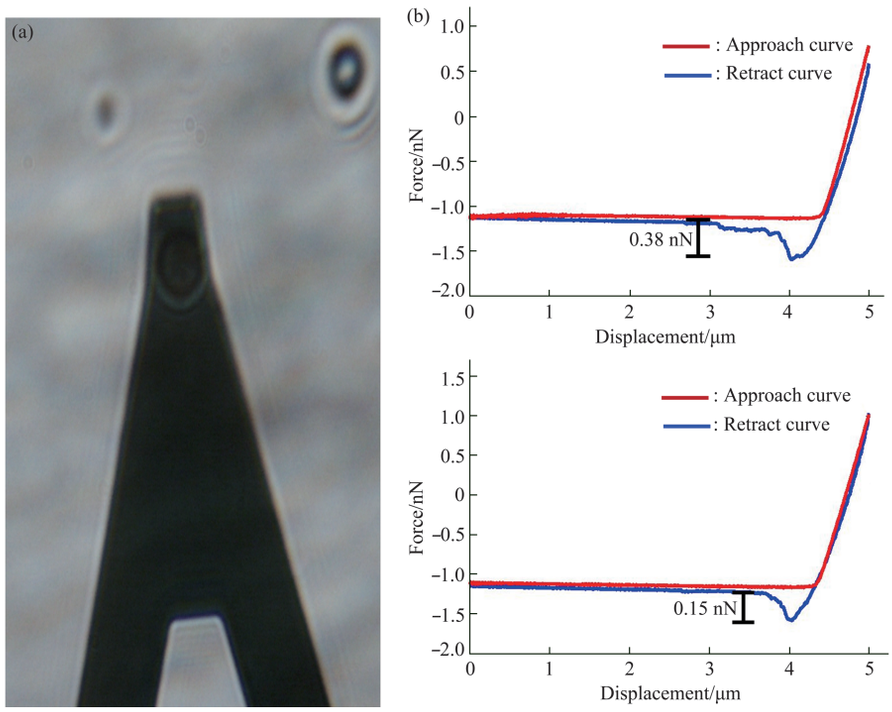

Fig. 3 Measuring the adhesion force between Raji cells and rituximab−coated substrate using single−cell force spectroscopy

（a） Optical image of controlling single-cell probe to perform approach-dwell-retract movements on the rituximab-coated substrate. （b） Representative force-distance curves recorded during the measurements. Force curves were recorded with contact time 1 s and loading force 1 nN. The measured detachment force is 0.38 nN for the top force curve and 0.15 nN for the bottom force curve respectively.

2 Results and discussion

2.1 Detecting the molecular adhesion force between Raji cells and BSA−coated substrate

We firstly measured the adhesion force between Raji cells and BSA-coated substrate for control experiments, as shown in Figure 4. The substrate of the Petri dish was coated by 50 mg/L BSA. Figure 4c is a typical force curve which clearly shows the force peak in the retract curve, indicating the adhesion between Raji cells and BSA. Figure 4a shows the results of the adhesion forces measured at different contact times between cell and substrate. We can see that the cellular adhesion force increased as the increase of contact times. However, when the contact time was larger than 2 s, the adhesion force measured by AFM largely kept stable (~ 0.35 nN) even the contact time further increased. Figure 4b shows the results of the adhesion forces measured at different loading forces between cell and substrate. We can see

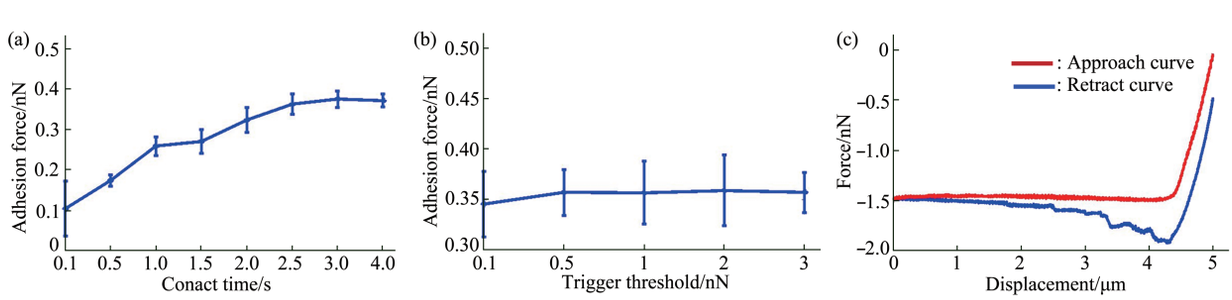

Fig. 4 The adhesion forces between Raji cells and BSA−coated substrate measured by AFM

（a） Cellular adhesion forces measured by varying the contact times between cell and substrate. （b） Cellular adhesion forces measured by varying the loading forces. （c） A typical force curve recorded during the measurements.

that on the whole the influence of loading force on the measured cellular adhesion force was weak and the cellular adhesion force kept stable when varying the loading forces.

2.2 Detecting the molecular adhesion force between Raji cells and rituximab−coated substrate

Figure 5 shows the adhesion force between Raji cells and rituximab-coated substrate measured with different concentrations of rituximab. Six concentrations of rituximab (0 mg/L, 20 mg/L, 40 mg/L, 60 mg / L, 80 mg / L, 100 mg / L) were used for coating the different sub-regions of the substrate of the Petri dish. The contact time of measurement is 1 s, and the trigger threshold is 1 nN. For each sub-region of the substrate, 100 force curves were recorded at 5 μm×5 μm areas. We can see that the adhesion force significantly increased from 0.145 nN to 0.699 nN when the concentration of rituximab increased from 0 mg / L to 60 mg / L. When the concentration of rituximab further increased, the adhesion force basically kept unchanged.

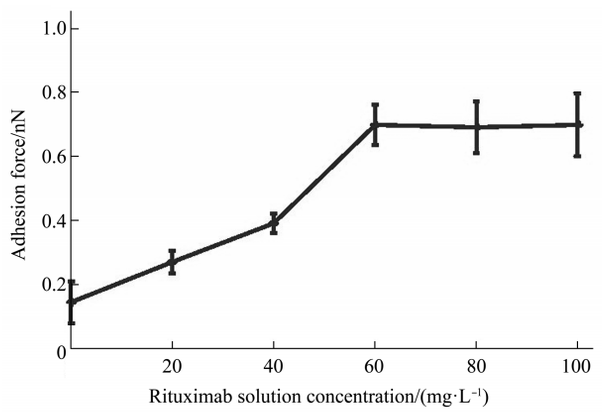

Fig. 5　The adhesion forces between Raji cells and rituximab−coated substrate measured by varying the concentrations of rituximab coated on the substrate

Figure 6 shows the adhesion force between Raji cells and rituximab-coated substrate measured with different contact times. The results of Figure 5 have shown that the suitable concentration of rituximab coated on the substrate for measuring adhesion force was 60 mg / L, when the trigger threshold is 1 nN. Then we measured the adhesion force on the substrate coated by 60 mg / L rituximab by varying the contact times between cell and substrate. As shown in Figure 6, eight different contact times (0.1 s, 0.5 s, 1 s, 1.5 s, 2 s, 2.5 s, 3 s, 4 s) were used for the measurements. When the contact time increased from 0.1 s to 1 s, the adhesion force rapidly increased from 0.367 nN to 0.668 nN. When the contact time increased to 2.5 s, the adhesion force slowly increased to about 0.763 nN and then kept stable.

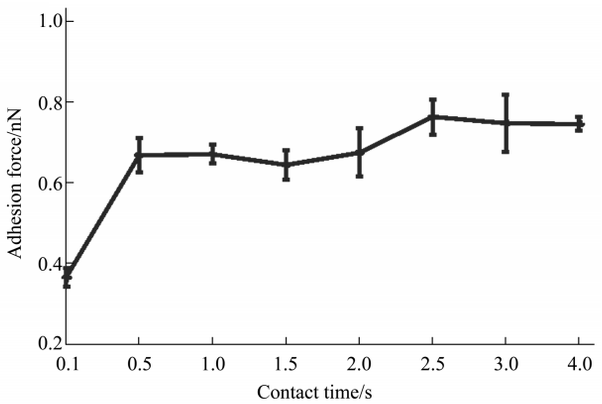

Fig. 6　Adhesion forces between Raji cells and rituximab−coated substrate measured by varying the contact times between cell probe and substrate

The substrate was coated by 60 mg/L rituximab.

Figure 7 shows the adhesion force between Raji cells and rituximab-coated substrate measured with different loading forces of AFM probe. According to the results in Figure 6, we used the contact time 1 s for measurements. From Figure 7, we can see that the adhesion forces nearly kept stable (~ 0.6 nN) when the loading force increased from 0.1 nN to 3 nN, indicating that the influence of loading force of AFM on the measurements of adhesion forces was weak.

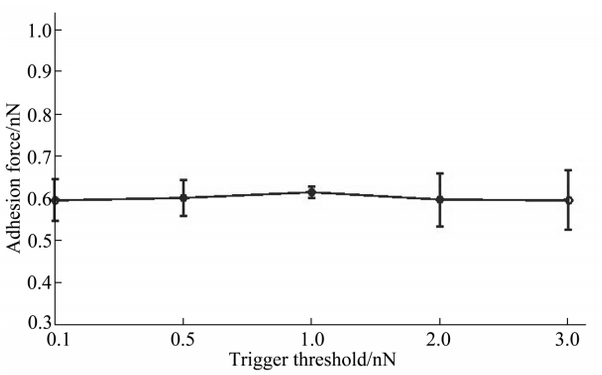

Fig. 7　The adhesion forces between Raji cells and rituximab−coated substrate measured by varying the loading forces

The substrate was coated by 60 mg/L rituximab.

Comparing the adhesion forces between Raji-BSA and Raji-rituximab, we can see that the adhesion forces between Raji cells and rituximab (~ 0.6 nN, Figure 7) were significantly larger than the adhesion forces between Raji cells and BSA (~ 0.35 nN, Figure 4). This is due to the different types of molecular interactions involved in these two types of adhesion. Rituximab specifically binds to the CD20 antigen on the Raji cell, which activates the signaling pathways for killing Raji cells[24], whereas the binding of Raji-BSA was due to the unspecific molecular interactions[25]. Therefore, the specific molecular binding of Raji-rituximab and the unspecific binding of Raji-BSA results in the different adhesion forces.

2.3 Detecting the cellular adhesion force between Raji cells

Figure 8 shows the adhesion forces between Raji cells. With the use of poly-L-lysine, single living lymphoma cells can be immobilized on the substrate[26]. By coating the substrate of Petri dish with poly-L-lysine, Raji cells were immobilized on the substrate. Under the guidance of optical microscopy, the single-cell probe was moved to a Raji cell (Figure 8a) and contacted the cell for the AFM-based SCFS measurements (Figure 8b). Adhesion forces between Raji cells were obtained by analyzing the recorded force curves. Figure 8c shows the adhesion forces measured by changing the contact times. We can see that the adhesion force increased from 0.161 nN to 0.457 nN when the contact time increased from 0.1 s to 4 s. Figure 8d shows the adhesion forces measured by changing the loading force of AFM probe. We can see that the variations of the loading force of AFM probe did not cause the significant changes of the adhesion forces between Raji cells. The results of Figure 8 showed the adhesive interactions between B lymphoma cells. On the surface of B-cell lymphoma cells, there are many different types of adhesion molecules, such as CD44 and CD24[27]. These adhesion molecules regulate the binding of Raji cells to perform various biological functions, which may cause the adhesive interactions between Raji cells. SCFS has been widely used to investigate cell adhesion, but these studies are commonly performed on adherent cells[28]. So far the information about the adhesive behaviors of single lymphoma cells is still scarce. Here, taking lymphoma cells as an example, our results prove the capabilities of AFM in detecting the cellular adhesive behaviors between human suspended cells, which will benefit the investigations of cell adhesion involved in the physiological and pathological changes of human suspended cells.

AFM-based SCFS provides a powerful tool for investigating molecular and cellular adhesive interactions. Traditionally, AFM measures molecular interactions by linking receptors onto AFM tip and then performing single-molecule force spectroscopy on cells[29-30]. The receptor-ligand interactions on the cell surface can then be probed. However, a disadvantage of this method is that it requires the biochemical functionalization of AFM tip, which is quite complex and time-consuming. Here, we directly attached single cell onto AFM cantilever and coated antibodies on the substrate, which allowed measurements of antigen-antibody molecular interactions. The method presented here was simple compared with traditional single-molecule force spectroscopy, thus providing a novel idea for investigating molecular interactions by AFM. Besides, we have expanded AFM-based SCFS to human suspended cells (lymphoma Raji cells) and the results showed the adhesive interactions between lymphoma cells. Detailed procedures were shown here to measure the molecular and cellular adhesion forces by AFM, including substrate treatment, cell-probe preparation, force measurements and analysis. The methods can be utilized to investigate the adhesive behaviors of other types of suspended cells, which will facilitate the studies of cell mechanics in tumor development and progression.

In summary, this work has demonstrated the use of AFM-based SCFS to quantitatively measure the molecular and cellular adhesive interactions taking place in lymphoma cells, which will potentially benefit the biomechanical studies for understanding the cell adhesion in cancer.

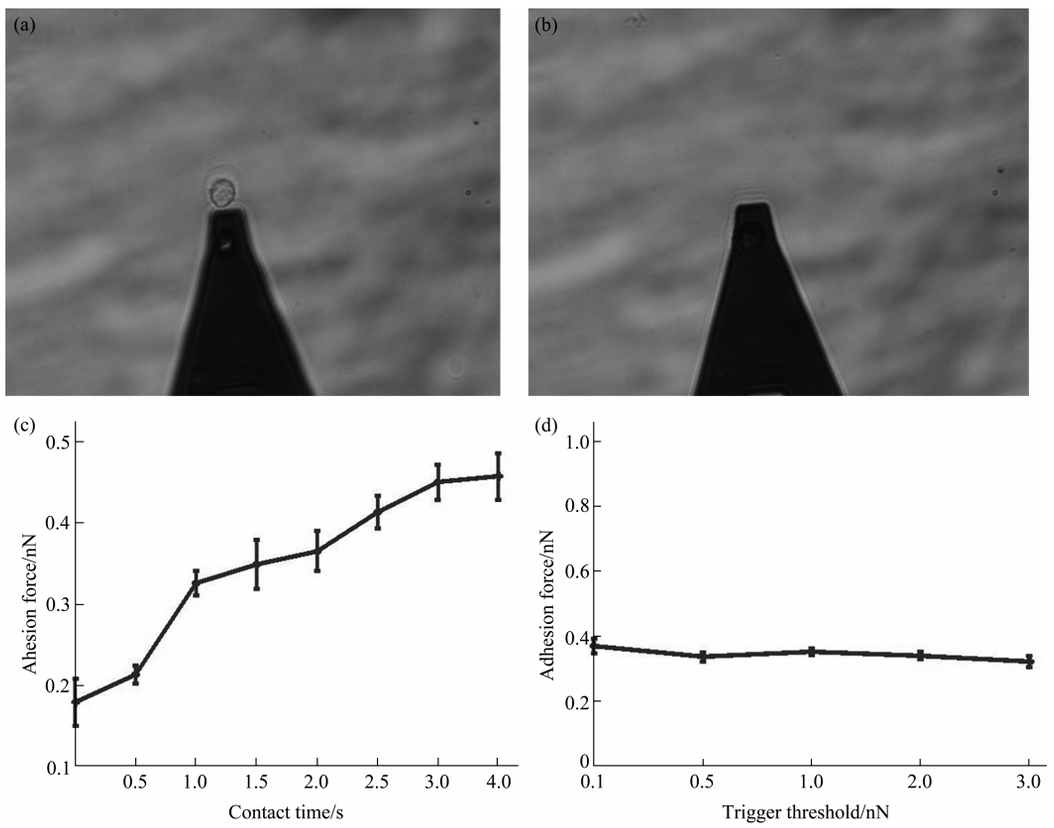

Fig. 8　The adhesion forces between Raji cells

One Raji cell was attached to AFM cantilever and one Raji cell was immobilized on the substrate. The adhesion forces between the two Raji cells were measured by AFM single-cell force spectroscopy. （a） The single-cell probe gradually approached individual Raji cell. （b） The single-cell probe contacted the Raji cell to perform measurements. （c） The adhesion forces between Raji cells measured by varying the contact times. （d） The adhesion forces between Raji cells measured by varying the loading forces.

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基于AFM单细胞力谱技术的淋巴瘤细胞黏附力测量*

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摘要　细胞黏附在细胞生理功能中起着重要的调控作用，对细胞黏附行为进行定量研究有助于理解生命活动内在机制. 原子力显微镜（AFM）的出现为研究溶液环境下微纳尺度生物系统的生物物理特性提供了强大工具，特别是AFM单细胞力谱（SCFS）技术可以对单细胞黏附力进行测量. 但目前利用SCFS技术进行的研究主要集中在贴壁细胞，对于动物悬浮细胞黏附行为进行的研究还较为缺乏. 本文利用AFM单细胞力谱技术（SCFS）对淋巴瘤细胞黏附行为进行了定量测量. 研究了淋巴瘤细胞与其单克隆抗体药物利妥昔（利妥昔单抗与淋巴瘤细胞表面的CD20结合后激活免疫攻击）之间的黏附力，分析了利妥昔浓度及SCFS测量参数对黏附力的影响，并对淋巴瘤细胞之间的黏附力进行了测量. 实验结果证明了SCFS技术探测动物悬浮细胞黏附行为的能力，加深了对淋巴瘤细胞黏附作用的认识，为单细胞尺度下生物力学探测提供了新的可能.

关键词　原子力显微镜，单细胞力谱，细胞黏附，淋巴瘤细胞，利妥昔单抗

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