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Atomic force microscope equipped with confocal laser scanning microscope for the spectroscopic measurement of the contact area in liquid

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Abstract

We developed a new experimental system (CLSM/AFM) and made the spectroscopic measurement in the contact area in liquid possible. We tried to measure the solvent properties between an AFM probe and hydrophilic or hydrophobic substrate surface. A microbead was attached to the AFM probe and fluorescent molecules were introduced to this probe to clarify the solvent property. As a result, when the probe was contacted, the fluorescence intensity changed according to these modified surfaces of substrates. This CLSM/AFM system could clarify the solvent property between an AFM probe and substrate surfaces directly.

1. Introduction

The recent invention of scanning probe microscopes has made it possible for us to study the physical and topographic properties of solid surfaces significantly clearer [1–3]. Among such microscopes, the atomic force microscope (AFM) [4] has been applied to the study of non-conducting biological systems such as cell surfaces [5,6] and DNA [7] by imaging them as well as solid state physics and material sciences, because of no requirement of electric conductivity on the sample surfaces and its operation under liquid environment. The force curve measurement mode of the AFM is eagerly employed in the structural and functional studies of biological molecules, such as protein-protein interactions [8,9] and protein unfolding [10–12]. Now, if the optical spectra of the contact area, such as fluorescence or Raman scattering, can be measured simultaneously with the force curve measurement, we should be able to obtain valuable information, such as the solvent properties or change in the electronic state of molecules between an AFM probe and substrate surfaces. For this purpose, we developed a new experimental system (CLSM/AFM) by combining an AFM with a confocal laser scanning microscope (CLSM) and we made the spectroscopic measurement of the contact area possible in liquid.

In this Letter, we measured the solvent properties between an AFM probe and two types of chemically characterized surfaces, one hydrophilic and the other hydrophobic, when they were brought into contact using the CLSM/AFM system. It is well known that the solvent molecules between solid surfaces play an important role in interactions owing to electric double layer force or adhesion force [13,14]. Therefore, many studies have been reported for clarifying the relation between the interaction and the solvent. For example, AFM was applied to probe the electric double layer at a surface under potential control [15], and the electric double layer force or adhesion force between various characterized surfaces was measured under various solvent [16], various ionic strength [17] or pH [18] conditions. To our knowledge, however, there was no previous study measuring the force and the solvent property simultaneously. We presently tried to measure this, using fluorescent dyes attached only to the surface

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of the AFM probe, and we compared the fluorescence intensity when the AFM probe contacted the substrate surfaces with that when it removed for minute vertical distance from the substrate (non-contact). As a result, the fluorescence intensity changed according to the modified surface of substrate, so it was shown that our CLSM/AFM system and this experimental technique could clarify the solvent property in the contact area directly.

2. Experimental

Fig. 1 shows the schematic diagram of the CLSM/AFM system. This system was composed of an AFM (Smena liquid head, NT-MDT, Moscow, Russia) inserted in the open space above the sample stage of an inverted CLSM (Nanofinder, Tokyo Instruments, Tokyo, Japan). An Ar^+ laser (488 nm, Nippon Laser, Japan) was used as the excitation light source. A pin hole (60 μm diameter) was used for improving the spatial resolution (about 450 nm). Fluorescence spectra of samples were measured with a cooled CCD camera (Andor Technology, Belfast, Northern Ireland) equipped with a spectrograph ($f = 56$ cm, 200 grooves/mm grating). When we worked with the CLSM/AFM system, the contact area of the AFM probe with the substrate had to be found and the focus point of objective lens had to be adjusted precisely both in vertical and lateral directions. To perform this promptly, another optical system was prepared in this system (Fig. 1 dotted line). This optical system enabled us to adjust the measurement point of the CLSM to the contact area to an accuracy of 400–500 nm, in terms of both vertical and lateral directions. As the CLSM system has a spatial resolution higher than conventional optical microscopes, it can reduce background light that is emitted out of the contact area. Therefore, this CLSM/AFM system can measure the spatial spectroscopic properties of the contact area selectively.

Fig. 2 illustrates schemes of our current measurement system. First, an AFM probe was brought into contact at the arbitrary point. Next, the lateral measurement point of the CLSM system was adjusted at the contact area and the vertical measurement point was adjusted on the substrate surface with the W-lamp image. Finally, force-distance curve and fluorescence spectra were measured. The measurement point of the CLSM was fixed at the first adjustment point. The non-contact spectra were measured by the following method: The AFM probe in the Z-direction was being moved continuously and being brought close to the substrate surface. Then, the non-contact spectra were measured at the suitable point with detecting the DFL signal for confirming that the AFM probe had not been contacting. After that, when the AFM probe was contacted, the relative distance between the measurement point and the contact point was estimated roughly. Since the acquisition efficiency of the fluorescence would decrease if the probe were separated from the substrate surface too much, the spectroscopic measurement was performed in the position near from the substrate surface as much as possible (within about 200 nm). The contact spectrum was measured with the arbitrary DFL signal.

A carboxylated polystyrene microbead (Polybead Carboxylate Microspheres, $r = 5$ μm, Polysciences, Warrington, PA) was attached to the end of an AFM cantilever (NP-S, spring constant = 0.12 N/m, Digital Instruments, Santa Barbara, CA) using micro manipulators (MMN-1, MMO-202ND, and MN-153, Narisige, Japan) to increase the number of dye molecules in the contact area [19,20] for better spectroscopic measurement. Fluorescein-5-maleimide (Molecular probes, Eugene, OR) was used as fluorescent probe for clarifying solvent properties. It was covalently cross-linked to the carboxylated surface of the microbeads as described below. After attachment a microbead to an AFM cantilever, condensation of carboxyl group of the bead and

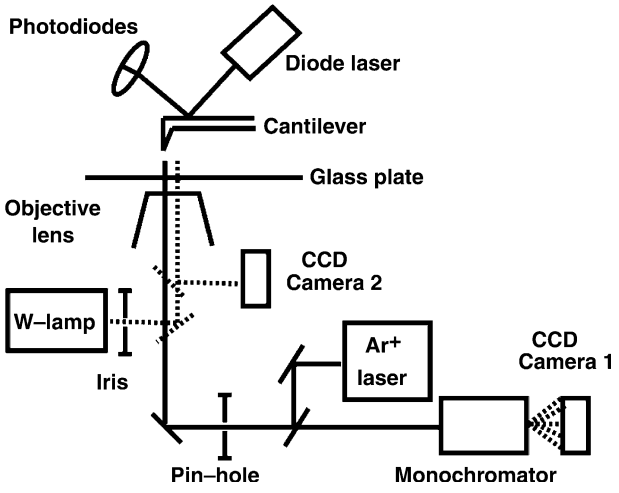

Fig. 1. The block diagram of the CLSM/AFM system.

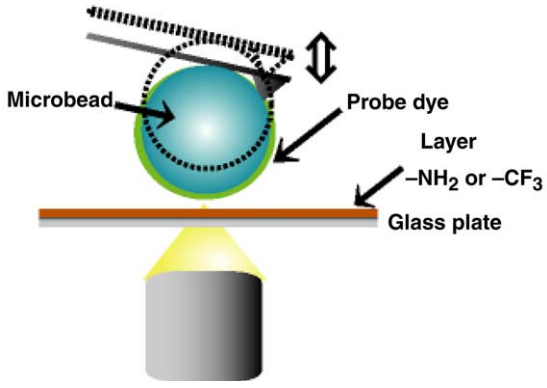

Fig. 2. The scheme of the present measurement.

N-hydroxysulfosuccinimide (Sulfo-NHS, Pierce, Rockford, IL) with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, Pierce, Rockford, IL) afforded active intermediate [21], which upon reaction with cysteine furnished SH terminated residue. The SH group was used for the introduction of fluorescein-5-maleimide. It is well known that fluorescein is a pH-sensitive dye and is more fluorescent in the H^+-dissociated form than in the H^+-undissociated form. Cover glasses were used as substrate after modification with 3-(diethoxymethylsilyl)propylamine (APDS) or dimethoxymethyl(3,3,3-trifluoropropyl)silane (FPDS) (Sigma, St. Louis, MO) by silane coupling method [22]. As the cover glass modified with APDS has a hydrophilic property on the surface due to the terminal functional groups of $–NH_2$. The cover glass modified with FPDS has functional groups of $–CF_3$, therefore, its surface has a hydrophobic property. Each substrate modified with each reagent has elastic properties similar to unmodified glass surface because the thickness of each modification layer is thin. In these experiments, we tried to investigate solvent properties in the contact area by measuring the change in the fluorescence intensity of fluorescein from contact state to non-contact state. In all experiments, the input power of excitation light was adjusted to 5 μW as measured after the pin hole and the liquid phase was kept at pH 9.5 with 100 mM carbonate buffered saline. Thus, fluorescein in bulk was in the H^+-dissociated form because of the alkaline and ionic condition.

3. Results and discussion

Fig. 3 shows a two-dimensional scattering image of an AFM probe with an attached microbead by irradiating with a W-lamp. The focus of the objective lens was adjusted to the upper surface of the cover glass and the AFM probe was brought into contact with the surface. The area on the glass surface where the spherical bead contacted looked circular, indicating that the center of this circle marked with an arrow was the contact area of the AFM probe.

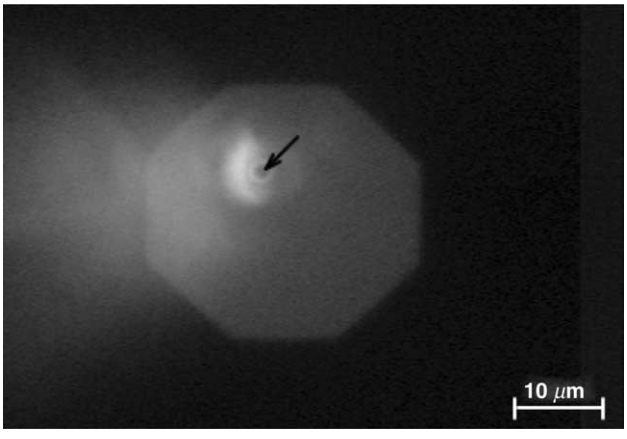

Fig. 3. Dimensional scattering image of a microbead attached to an AFM probe with a W-lamp. The AFM probe was brought into contact and the focus of objective lens was adjusted to the upper surface of cover glass by monitoring the octagonal iris image of W-lamp. The microbead looks like a large circle, and the area which spherical bead contacted looked circular.

At first, we observed the resonance energy transfer from the donor dyes immobilized AFM probe to the acceptor dyes attached to the substrate surface. As a result, it was clarified that the fluorescence of the acceptor and donor was enhanced and quenched, respectively, and that the resonance energy transfer occurred in the center of this circle only (data not shown). Therefore, it was confirmed that the center of this circle was the contact area and the signal of the contact area could be measured in this present experimental condition by our CLSM/AFM system.

Solid lines in Figs. 4a and b show fluorescence spectra of the contact area when the AFM probe was in contact with substrate surfaces having terminal groups of $–NH_2$ and $–CF_3$, respectively. The vertical positions of the AFM are shown by solid circles in the figures. The dotted line shows the fluorescence spectrum when it was in non-contact. Here, the positions of the AFM probe are shown by open circles. These results showed that fluorescein in the contact area was not quenched by the $–NH_2$ surface but was quenched by the $–CF_3$ surface. Back ground light such as fluorescence from the unmodified bead or modified glass surface was not observed in these experimental conditions (see Fig. 4a thin line). And no fluorescence intensity change was seen when the probe was in contact with an unmodified surface (data not shown). The fluorescence intensity

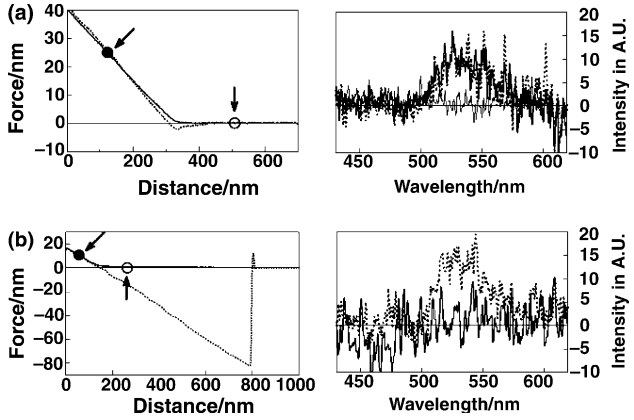

Fig. 4. Force–distance curves and fluorescence spectra of the contact area. Panels (a) and (b) show results of $–NH_2$ and $–CF_3$ terminated surfaces, respectively. The spectra shown by solid and dotted lines were taken at the distances shown by solid and open circles in force-distance curve, respectively. The baselines of these spectra were defined as the noise including the dark current of the detector and stray light from out of the experimental system. Background level (such as fluorescence of the unmodified bead, etc.) is shown by thin line in (a).

ordinarily decreased due to photobleaching of the fluorescent dye. Therefore, in order to discuss about this qualitatively and quantitatively, the effect of the photobleaching need to be considered. The fluorescence spectra of the contact area were measured by applying a fixed load about 5 nN by repeating contact and non-contact (within about 200 nm above the substrate) alternately. The integrated fluorescence intensity in the 500–600 nm regions was summarized in Fig. 5. Figs. 5a and b show typical results for the measurements of $-NH_2$ terminated surfaces and $-CF_3$ terminated surfaces, respectively. In these figures, the vertical axis shows the integrated fluorescence intensity and the horizontal axis corresponds to the sequential number of spectroscopic measurement from 1 (exposure time 1 s, excitation light 5 μW). In Fig. 5, solid and open circles represent data obtained with the AFM probe in contact and in non-contact (within about 200 nm), respectively. The measured fluorescence intensity decreased in both cases due to the photobleaching of the dyes. These photobleaching curves exhibited different temporal profiles from one another even if the same sample was used. The difference between two photobleaching curves in Fig. 5 is not due to the background noise or difference in the modified surface but due to the difference in the effect of oxygen on the degradation of dyes. Thus, we alternately measured the fluorescence intensity in contact and in non-contact condition and considered the difference between them. Although fluorescence intensity is not significantly different either in contact or in non-contact condition for the $-NH_2$ terminated surfaces, Fig. 5b shows that for $-CF_3$ terminated surfaces the fluorescence intensity in contact was remarkably weaker than in non-contact. In this result, it can be confirmed that the quenching of the fluorescence is not due to the photobleaching but the probe contact with the hydrophobic surface. Although the fluorescence spectra in Fig. 4 were measured when the fluorescence of the dye molecules was stable for the photobleaching (see Fig. 5) and we show an example for the perfect quenching of the fluorescence in Fig. 4, the efficiency of the quenching was ordinarily fluctuated. If the substrate surface has a high compliance, the contact area should increase as the probe pushes in the substrate surfaces [23,24]. However, since both the polystyrene microbeads and the glass substrates are quite hard. It is considered that the contact area between the microbead and the glass surfaces have been still smaller than the region monitored by CLSM. Therefore, the fluorescence from the dyes in bulk was also measured. Similar experiments were repeated, and the ratio of the intensity of contact to that of non-contact was statistically calculated to be 70 (±20)%. We assume that this value showed the rate of the contact area to the fluorescence measurement region. However, this value is meaningful only in this measurement system. In order to give a quantitative and scientific meaning, it is necessary to perform comparison with same well-defined samples.

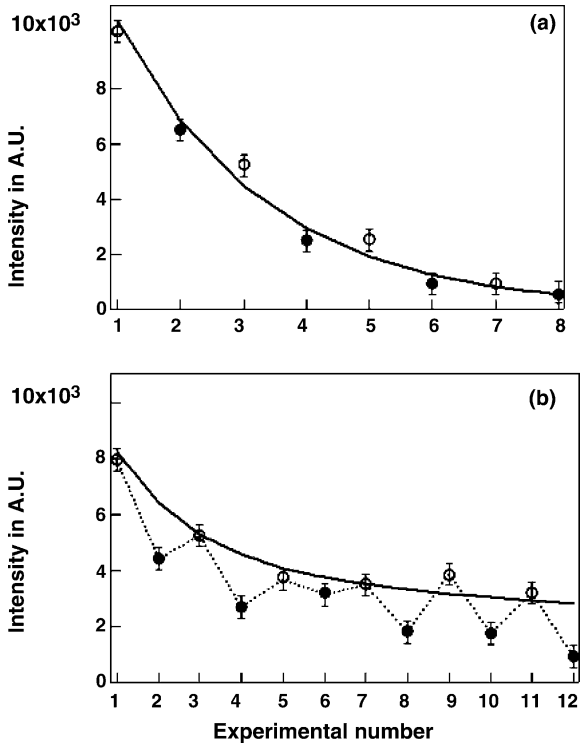

Fig. 5. Integrated fluorescence intensity of fluorescein (500–600 nm) on the surface of an AFM probe. Panels (a) and (b) show typical results for the measurements of $-NH_2$ surfaces and $-CF_3$ surfaces, respectively. In this figure, solid or open circle represents a data which was obtained when an AFM probe was contacted by applying the load of 5 nN or separated from the surface within about 200 nm, respectively. The dotted line is a photobleaching curve created on the basis of open circle. The error bars were statistically calculated by integrating (500–600 nm) the data measured only the background noise.

It is considered that why the quenching occurred is because the solvent molecules between the AFM probe and the $-CF_3$ surface escaped when the probe was brought into contact and the fluorescence quantum yield of fluorescein decreased because fluorescein in the contact area became H^+-undissociated form which is stable in a hydrophobic environment. On the contrary, on an $-NH_2$ terminated surface, it is considered that solvent molecules remained in the contact area and fluorescein molecules were in the H^+-dissociated form. Fig. 4b shows that a strong adhesion between the probe and the surface was observed only on a $-CF_3$ terminated surface. A carboxylated polystyrene microbead has both the hydrophilic ionizable –COOH groups and the hydrophobic polystyrene surfaces. Even if all the carboxyl groups were ionized ($-COOH \rightarrow -COO^-$) in the alkaline condition, the bead adhered to the hydrophobic $-CF_3$ terminated surface (data not shown). These results indicate that number of –COOH on the bead is insufficient to make the surface hydrophilic completely. Although a fluorescein molecule attached to the bead has a ionizable

–COOH and an aromatic –OH, the surface of the bead still showed hydrophobic property. Therefore, it is considered that the strong adhesion shown in Fig. 4 is due to the hydrophobic interaction. On the other hand, $–NH_2$ groups are not charged ($–NH_2 \rightarrow –NH_3^+$) in this experimental condition (pH 9.5 aqueous solution [18]). So it is considered that electrostatic force between charged groups, hydrogen bonding between –COOH (or –OH) and $–NH_2$ groups, and hydrophobic force (between hydrophobic surfaces) may be weak in this experimental condition.

Therefore, it is concluded that the solvent molecules are removed and the charged groups are compulsorily altered to their neutral forms between surfaces when the hydrophobic interaction is occurred.

In this study, it is shown that the solvent properties between an AFM tip and the substrate surfaces can be directly probed with a CLSM/AFM system and the experimental technique presented in this Letter. We could observe that the solvent molecules were removed when a hydrophobic interaction was occurred. Our experimental method has a potential capability to reveal a mechanism of interactions between solid surfaces in liquid by measuring solvent property between various surfaces or in various solvent conditions. This CLSM/AFM system and our experimental method will be applied to the various experiments with improving the system accuracy and the performance.

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