Paper

2D Visualization of Mannose Distribution Using AFM Force Sensing with Probe Modified by Concanavalin A

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It is very important to clarify the distribution of sugar chains on the surface of biological membranes for understanding physiological reactions in detail. However, there has been little information available on the two-dimensional distribution of sugar chains on biological surfaces on micro- to nanometer scale until now. Atomic force microscopy (AFM) has been widely used for the direct detection of specific interactions between biologically active molecules. *Concanavalin A (ConA)* is a well-known lectin with specific affinities for α-D-mannosyl and α-D-glucosyl residue. In this study, mannose (Man) and oligoethylene glycol (OEG) were locally immobilized on a substrate, and the two-dimensional distribution was visually mapped on micrometer scale using AFM force sensing with a *ConA*-modified tip. As a result, the distinct contrast between Man and OEG regions was observed based on the difference of the interaction with *ConA*. It is expected that distribution analysis of sugar chains on biological membranes will be realized by this method in the near future.

1. Introduction

It is very important to clarify the distribution of sugar chains on the surface of biological membranes for understanding physiological reactions such as virus infection [1]. However, there has been little information available on the two-dimensional distribution of sugar chains on biological surfaces on micro- to nanometer scale due to the complexity and diversity until now.

Atomic force microscopy (AFM) is a method for high-resolution imaging of surface structures based on interactions between a surface and a sharp AFM tip [2]. AFM can be also used to measure interaction forces between biologically active molecules. In these measurements, a biomolecule is attached to an AFM tip, and it is used to probe a surface modified with a comprehensive biomolecule. Recently, such functionalized AFM tips have been expected as a powerful probe for measuring the binding properties of biological interactions at the single molecule level on naturally-occurring surfaces such as biological membranes or artificial surfaces such as ligand-functionalized membranes [3, 4, 5].

Lectins are proteins that can recognize a sugar chain and have been utilized as very useful probes in studying sugar chains on cell surfaces based on their specific recognition abilities [1]. In particular, *concanavalin A (ConA)* is a well-known lectin with specific affinities for α-D-mannosyl and α-D-glucosyl residues [6]. The interaction between *ConA* and corresponding simple sugars has been previously studied by AFM [7, 8, 9]. However, studies on the visualization of the two-dimensional distribution of sugar chains by AFM force sensing with a *ConA*-modified tip are still challenging.

Our purpose is to establish a simple method to visualize the two-dimensional distribution of sugar chains on biological membranes using AFM directly. In this study, the mannose (Man)- and oligoethylene glycol (OEG)-terminated pattern substrate was fabricated, and its two-dimensional distribution was visually mapped on micrometer scale by AFM force sensing based on the specific interaction force between Man and *ConA*.

2. Experimental

2.1. Materials

Alkanethiols with an oligoethylene glycol

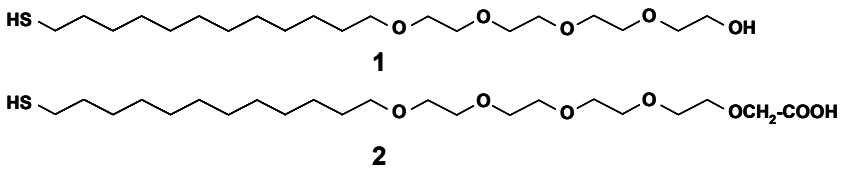

Fig.1. Molecular structure of thiols used in this study.

moiety; tetraethylene glycol-dodecane thiol (**1**) and acetic acid-tetraethylene glycol-dodecane thiol (**2**) were prepared according to the previous published paper (Fig.1) [10, 11]. Highly purified *concanavalin A*, D-(+)-mannose, ammonium carbonate, *N*-hydroxysuccinimide (NHS) and water-soluble carbodiimide hydorochloride (WSC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amination of mannose was carried out by the Kochetkov reaction [12]. Phosphate-buffered saline (PBS, pH 7.4) was purchased from Takara Bio (Tokyo, Japan). Deionized water was prepared using a Milli-Q system with the resulting water having a conductivity of 18 M Ω cm^{-1}.

2.2. AFM measurements

Gold-coated AFM tips with a nominal spring constant of 0.025 N/m (Olympus, Tokyo, Japan) were used for force measurements. Spring constants were calibrated in the range of 0.022-0.028 N/m using the thermal noise method. All force measurements were performed in a PBS solution (pH 7.4) using a NanoScope V Multi Mode AFM (Veeco, Santa Barbara, CA). For all force measurements, a contact force of 200 pN, ramp size of 200 nm, and surface delay of 3 s were used. Force-curve data were collected by the force-volume technique as described previously [13]. This method allows force curves to be acquired as a function of the lateral position on the sample surface. A complete force curve was recorded at each position while the AFM tip was raster scanned across the surface of the sample over a 64 × 64 point array. Height images (64 × 64 pixels) were recorded simultaneously by the force-volume mapping. Following acquisition, AFM force-volume data was analyzed offline with a purpose-built program developed in our laboratory based on Microsoft Visual Basic 6.0.

2.3. Fabrication of Man- and OEG-terminated pattern substrate

As alkanethiols spontaneously form self-assembled monolayers (SAMs) on a gold substrate, several methods have been reported for constructing SAMs containing sugar chains on gold substrates. The typical method is based on the direct chemical modification of SAMs [14, 15]. In this method, sugar moieties were introduced into the functionalized SAMs with reactive sites using the chemical reactions. The fabrication of "patterned" SAMs has been demonstrated on a gold substrate by a colloidal lithography technique using silica nanoparticles as a mask [16, 17]. Briefly, the patterned substrate was fabricated as follows (Fig.2). Firstly, a gold-coated (50 nm) silicon substrate (1) was immersed into an ethanol solution containing 1 mM **1** for 24 hours (2). The resulting SAM was rinsed with ethanol and dried with nitrogen. Next, mica pieces were deposited on the substrate (3). The typical size of the mica pieces was 10-20 μm in width and length. After deposition of mica pieces, a gold layer was deposited onto the substrate by vacuum evaporation again (4) and immersed into an ethanol solution containing both 1 mM **1** and **2** (1:1) for 24 hours, before subsequent immersion for 30 min into a solution of 0.1 M WSC and 0.1 M NHS in deionized water. Following the activation of the terminal COOH, the substrate was rinsed with deionized water, dried with nitrogen and then mannosylamine was introduced into the surface by the condensation reaction (5). Finally, the mica pieces were removed from the substrate under running water (6).

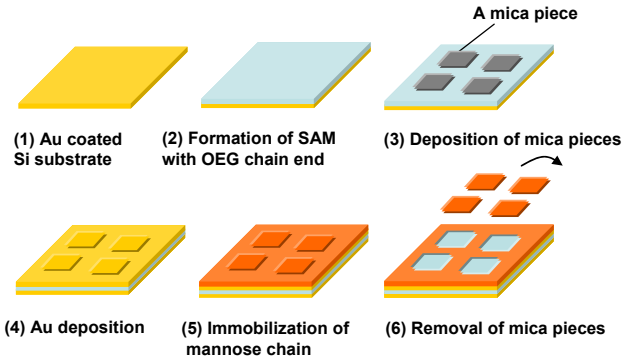

Fig.2. Schematic diagram of fabrication of patterned substrate using mica pieces.

2.4 AFM tip modification

The gold-coated AFM tip was immersed into an ethanol solution containing 1 mM **2** for 24

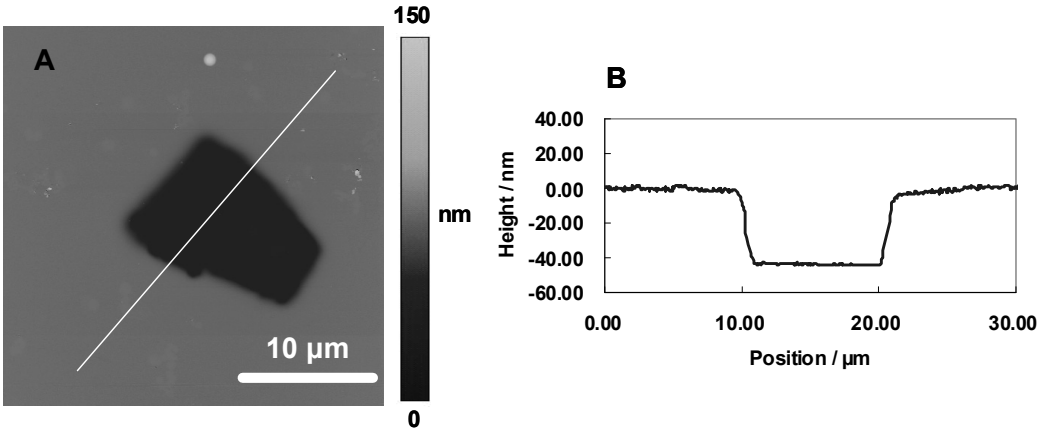

Fig.3. (A) AFM topographic image of the Man- and OEG-terminated pattern substrate. The higher and lower parts were the Man- and OEG-terminated regions, respectively. (B) Cross-section profile of the patterned substrate along the white line shown in (A).

hours and then rinsed with ethanol and dried with nitrogen. The tip was then immersed for 30 min into an aqueous solution containing 0.1 M WSC and 0.1 M NHS. Following the activation, the tip was rinsed with deionized water, and dried with nitrogen. Finally, the NHS-activated AFM tip was immersed into a PBS solution (pH 7.4) containing 0.1 mg/ml *ConA* for 2 hours, and rinsed again with the PBS solution.

3. Results and discussion

Fig.3 shows AFM topographic images of the Man- and OEG-terminated pattern substrate. In the AFM topographic image, the bright and dark parts correspond to the higher and lower regions of the monolayer surface, respectively. As expected, the higher and lower regions in the topographic image corresponded to the Man- and OEG-terminated SAMs, respectively. Also, the flat depression of the topographic pattern was approximately 40 nm in depth.

Fig.4 shows typical force curves obtained from the Man- and OEG-terminated regions using the *ConA*-modified AFM tip. In the OEG-terminated region, any adhesion force was rarely observed in the retraction force curve, as seen in Fig.4A. It is well known that the use of an OEG-terminated thiol is an effective procedure to significantly reduce the non-specific adhesion interactions with proteins [18]. Our results are consistent with the previous investigations. In the case of the Man-terminated region, the multiple adhesion forces, which are distinguishable from the non-specific adhesion forces between *ConA* and OEG, were observed in the retraction force curve (Fig.4B). At pH 7.4, where *ConA* exists as a tetramer, multivalent interactions seem to be apparent as indicated by Zhang et al [19]. By contrast, characteristic forces were not observed even in the Man-terminated region using an unmodified AFM tip.

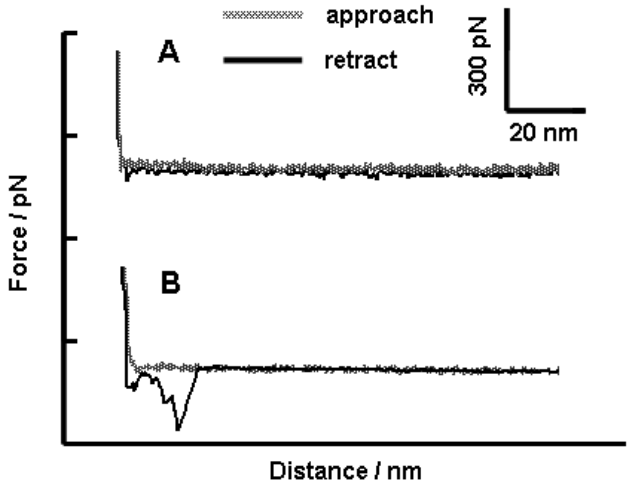

Fig.4. Typical AFM force curves obtained from (A) OEG-terminated, and (B) Man-terminated regions.

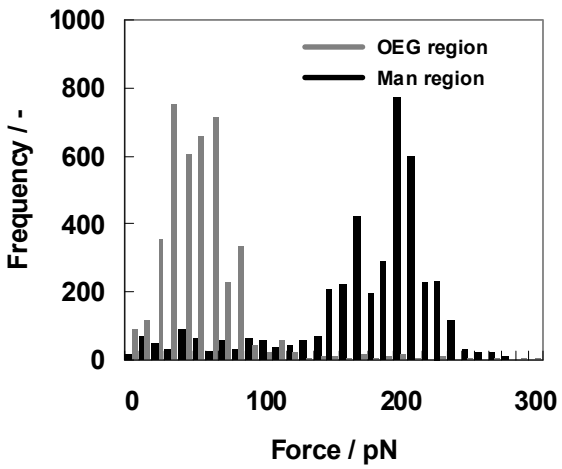

Fig.5. Histogram of adhesion forces obtained from Man- and OEG-terminated regions.

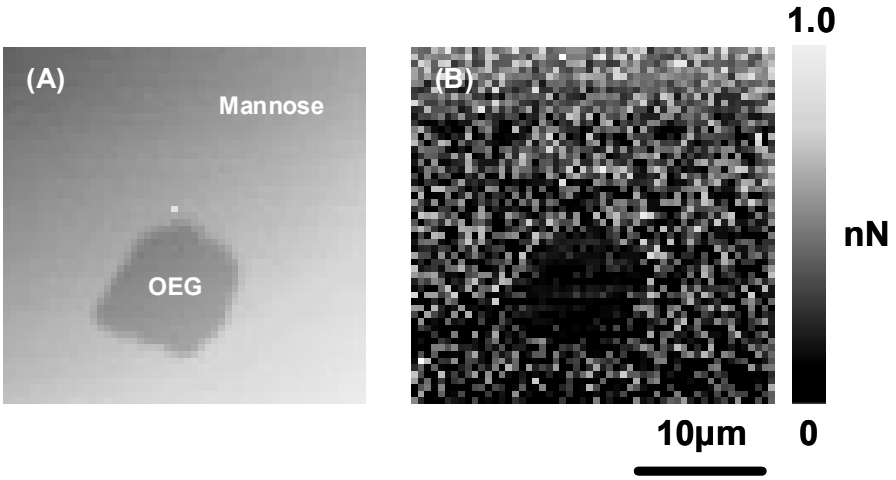

Fig.6. (A) AFM topological image and (B) adhesion force image of the Man- and OEG-terminated pattern substrate obtained with a *ConA*-modified tip.

The histogram of adhesion forces is shown in Fig.5. In the Man-terminated region, the specific adhesion force was the center of peaks around 200 pN (194.5 ± 25.3 pN). On the other hand, in the OEG-terminated region, the non-specific adhesion force was the center of peaks around 50 pN (53.1 ± 10.5 pN). As a result, it was confirmed that the Man- and OEG-terminated regions were fully distinguishable by using the AFM force sensing. Fig.6 shows AFM topological and adhesion force images of the Man- and OEG-terminated pattern substrate obtained with a *ConA*-modified tip. A sharp contrast between the Man- and OEG-regions was clearly observed on micrometer scale in the adhesion force image, and the pattern was in a good agreement with that of the topographic image. Generally, it has been difficult to detect the interaction forces between *ConA* and Man by AFM because of the weak interaction forces (~200 pN). However, it was possible to visualize the Man region by AFM force sensing using the controlled sugar-modified substrate and the *ConA* -modified tip, here. As a further application, this method was applied to a model biological surface comprised of phosphorylcholine (PPC)- and Man-terminated SAMs. As a result, it was also able to selectively visualize the Man region on the PPC- and Man-terminated pattern surface. These results suggest that our method is broadly applicable to a variety of biological surfaces.

4. Conclusion

In conclusion, we have successfully visualized the two-dimensional distribution of sugar chains on the model biological surface by AFM force sensing with a *ConA*-modified tip. Thus, the distribution analysis of sugar chains on biological membranes or cell surfaces will be realized by this method in the near future.

5. References

[1] A. Varki, *Glycobiology* **3**, 97 (1993).
[2] G. Binnig, C. F. Quate, C. Gerber, *Phys. Rev. Lett.* **56**, 930 (1986).
[3] U. Dammer, M. Hegner, D. Anselmetti, P. Wagner, M. Dreier, W. Huber, H. J. Guntherodt, *Biophys. J.* **70,** 2437 (1996).
[4] P. Hinterdorfer, Y. F. Dufrene, *Nat. Methods* **3**, 347 (2006).
[5] F. Kienberger, G. Kada, H. Mueller, P. Hinterdorfer, *J. Mol. Biol.* **347**, 597 (2005).
[6] H. Lis, N. Sharon, *Chem. Rev.* **98**, 637 (1998).
[7] M. Lekka, P. Laidler, J. Dulinska, M. Labedz, G. Pyka, *Eur. Biophys. J. Biophys. Lett.* **33**, 644 (2004).
[8] A. Touhami, B. Hoffmann, A. Vasella, F.A. Denis, Y.F. Dufrene, *Langmuir* **19**, 1745 (2003).
[9] T. V. Ratto, K. C. Langry, R. E. Rudd, R. L. Balhorn, M. J. Allen, M. W. McElfresh, *Biophys.J.* **86**, 2430 (2004).
[10] M. Tanaka, T. Sawaguchi, Y. Sato, K. Yoshioka, and O. Niwa, *Tetrahedron Lett.* **50**, 4092 (2009).
[11] Y. Nakatsuji, M. Muraoka, M. Wada, H. Morita, A. Masuyama, T. Kida, I. Ikeda, *J. Org. Chem.* **62**, 6231 (1997).
[12] L. Likhosherstov, O. Novikova, V. A. Derveitskaja, N. K. Kochetkov, *Carbohydr. Res.* **146**, c1 (1986).
[13] M. Radmacher, J. P. Cleveland, M. Fritz, H. G. Hansma, P. K. Hansma, *Biophys. J.* **66**, 2159 (1994).
[14] T. P. Sullivan, W. T. S. Huck, *Eur. J. Org. Chem.* 17 (2003).
[15] S. Flink, F. C. J. M. van Veggel, D. N.

Reinhoudt, *Adv. Mater.* **12**, 1315 (2000).
[16] M. Akiyama, M. Fujita, M. Fujihira, *Chem. Lett.* **35**, 1112 (2006).
[17] S. Kado, H. Yano, Y. Nakahara, K. Kimura *Chem. Lett.* **38**, 58 (2009).
[18] C. Pale-Grosdemagne, E. S. Simon, K. L. Prime, G. M. Whitesides, *J. Am. Chem. Soc.* **113**, 12 (1991).
[19] X. Zhang, V. K. Yadavalli, *Anal. Chim. Acta* **649**, 1 (2009).