A nano-scale probing system with a gold nano-dot array for measurement of single biomolecular interaction force

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Experimental Section

Fabrication of Au Nano-dot Array. Various sizes of Au nano-dots from 20 nm to 70 nm by units of 10 nm in diameter and approximately 20 nm in height were fabricated on a silicon substrate by a micromachining process (Fig. 1A in main manuscript). Briefly, after coating a photoresist (PR) on the silicon (Si) wafer, nano-dot patterns were lithographed on the PR using an electron beam (E-beam). Then, Cr and Au were deposited in series using an evaporation technique. Finally, the PR was removed from the Si wafer using a lift-off process, and the Au nano-dots of various sizes were acquired. A macro-scale marker was patterned near the array using aluminum because the exact location of the nano-dots was difficult to identify using the vision camera of the probing instrument. Nine dots of equal diameter were grouped together, and 6 patterns of the same size were designed for each group on the substrate. The central distance between the dots was 250 nm. The sizes of the dots were determined by considering the size of the target protein, which is expected to be several nanometers. In addition, Au was used for the dot material due to the resulting easy immobilization of proteins.

Laboratory-made Probing System. To measure the unbinding force between a carbohydrate and a single protein, a probing instrument adopting the working principle of atomic force microscopy (AFM) was developed. The instrument was composed of ultra-precise manipulation stages, an optical force detector, a data acquisition and feedback controller, and an optical microscope with a CCD camera (Fig. 2A in main manuscript). The detailed measurement technique of the proposed method is identical to that of AFM for obtaining a force-distance (F-D) curve. In AFM, because finding the selected surface profile on a substrate by scanning a large area generally takes a long time, it is difficult to select a starting position. Therefore, our instrument was equipped with 1500×x magnifying optics.
(DZ2; Union Optical, Tokyo, Japan) and a high-resolution digital CCD camera (Infinity lite; OPT, CA, USA) to recognize the macro-scale patterns for determining the starting position of measurements around the nano-dots, which are rarely discernable. The movement components of the tip were composed of feedback-controlled 3-axis motorized stages (LTA-HS & M-462-XYZ-SD, Newport, CA, USA) and piezo-driven 3-axis fine stages (NanoCube; Physik Instrument, Karlsruhe, Germany), which possess 0.5 μm and 2 nm closed-loop resolution, respectively. From the combination of the two types of stages, we were able to locate the substrate (nano-dot array) at the proper starting position in a short time. The optical lever force detector was composed of a 1-dimensional position-sensitive detector (PSD; S3931; Hamamatsu, Shizuoka, Japan) with a signal processing board (C3683-01) and laser diode and optics (Edmund Optics, NJ, USA). It was designed to detect an up to 0.1 nm vertical deflection of the probe cantilever to detect a force below 5 pN (Fig. 2B in main manuscript).

**Carbohydrate and Protein Preparation.** Aminophenyl disulfide, dimethylamino borane, and GM1 pentasaccharide were purchased from Sigma-Aldrich (Saint Louis, MO, USA), Fluka (Saint Louis, MO, USA), and Enzo Life Sciences (Plymouth Meeting, PA, USA), respectively. Coupling reagents for carbohydrate and protein immobilization consisted of the following: (3-aminopropyl)-triethoxy silane from Sigma-Aldrich, 8-amino-1-octanethiol N-succinimidyl 3-(2-pyridyldithio)propionate and succinimidyl octanoate (dithiobis) from Dojindo (Kumamoto, Japan), and MAL-dPEG<sub>24</sub><sup>TM</sup>-NHS ester from Quanta Biodesign (Powell, OH, USA). HBS-EP buffer (10 nM HEPES (pH 7.4), 10 mM NaCl, 3 mM EDTA, and 0.005% surfactant F20) and dithiothreitol (DTT) were purchased from GE Healthcare (New York, NY, USA) and USB Corp. (Cleveland, OH, USA). *V. cholerae* toxin B subunit protein was purchased from Sigma-Aldrich.

Carbohydrate modification followed the same protocol as described in a previous work.26
Briefly, 50 mM GM1 pentasaccharide and 50 mM aminophenyl disulfide were dissolved in water and glacial acetic acid, respectively. Then, the carbohydrate and aminophenyl disulfide solutions were mixed sufficiently and incubated in sealed tubes for 1 h at 30 °C for coupling reaction. To quench the reaction, 100 mM dimethylamine borane (freshly prepared) was added to each reaction, and the tubes were incubated for 1 h at room temperature. Each product was evaporated under nitrogen gas streaming for 1 h at 50 °C for condensation. Finally, the modified carbohydrates were dissolved in a proper solution (distilled water or HBS-EP buffer) and preserved at -20 °C in dark conditions before immobilization onto an AFM cantilever.

**Protein Immobilization on Patterned Nano-dot Array.** The Au nano-dot array was immersed into 1 mM dithiobis. Then, after dipping the array in acetonitrile for 1 h, the nano-dot surface was crosslinked with *V. cholerae* toxin B subunit protein (20 μg/mL) after cleaning with HBS-EP buffer before the force measurement.

**Cantilever Modification for Carbohydrate Immobilization.** The silicon nitride (Si$_3$N$_4$) cantilever probes (NP20; Veeco, Santa Barbara, CA, USA) were cleaned in an O$_2$ plasma for 1 h and then transferred into (3-aminopropyl)-triethoxy silane (Sigma) in the saturated condition for 12 h, resulting in an aminosilane-functionalized probe tip. The aminosilane cantilevers were then functionalized with 1 mM of MAL-dPEG$_{24}$TM NHS ester (Quanta Biodesign, Powell, OH, USA) in HBS-EP buffer for 1 h. Finally, after washing twice with HBS-EP buffer, the thiol group-modified GM1 pentasaccharide was treated for 2 h. To prevent the drying out of the linker and carbohydrate solution, the modified probes were incubated in a humidity chamber.

**F-D Curve Measurement.** The unbinding force measurement was conducted using the functionalized cantilever probe and protein-immobilized Au nano-dot array. As previously mentioned, the starting position is important for the measurement. If the starting position is
viewed from the top, the end of the probe can be easily located on the micro-markers. In addition, the nano-dots can be easily located due to the closed-loop controlled ultra-precise dual-stages. However, the probe location is not directly visible because it is on the bottom side of the probe. Therefore, a micro-scale was used to measure the relative distance from the end of the probe to the center position of the tip when it is viewed from the top (Fig. 2B in main manuscript). As a result, the starting position of the measurement was appropriately located without scanning the substrate. Then, the unbinding force was measured around the nano-dots. The stiffness of the functionalized cantilever probe was 0.06 N/m (from supplier datasheet), and the scanning speed of the probe was set to 100 nm/sec as a constant loading rate.