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Functionalized self-assembled monolayers for measuring single molecule lectin carbohydrate interactions

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ABSTRACT

The specific interactions between sugar-binding proteins (lectins) and their complementary carbohydrates mediate several complex biological functions. There is a great deal of interest in uncovering the molecular basis of these interactions. In this study, we demonstrate the use of an efficient one-step amination reaction strategy to fabricate carbohydrate arrays based on mixed self-assembled monolayers. These allow specific lectin carbohydrate interactions to be interrogated at the single molecule level via AFM. The force required to directly rupture the multivalent bonds between Concanavalin A (Con A) and mannose were subsequently determined by chemical force microscopy. The mixed self-assembled monolayer provides a versatile platform with active groups to attach a 1-amino-1-deoxy sugar or a protein (Con A) while minimizing non-specific adhesion enabling quick and reliable detection of rupture forces. By altering the pH of the environment, the aggregation state of Con A was regulated, resulting in different dominant rupture forces, corresponding to di-, tri- and multiple unbinding events. We estimate the value of the rupture force for a single Con A–mannose bond to be 95 ± 10 pN. The rupture force is consistent even when the positions of the binding molecules are switched. We show that this synthesis strategy in conjunction with a mixed SAM allows determination of single molecules bond with high specificity, and may be used to investigate lectin carbohydrate interactions in the form of carbohydrate arrays as well as lectin arrays.

1. Introduction

Biomolecular recognition between receptors and their cognate ligands is a key step in a wide variety of important biological processes. In particular, cell-surface carbohydrates act as important biomarkers in the process of molecular recognition and the regulation of a multitude of complex biological functions [1]. The specific interactions between sugar-binding proteins (lectins) present on cell-surfaces, and their complementary carbohydrates mediate various processes in living organisms such as cell–cell recognition, cellular adhesion and signaling events at the cell-surface [2]. Carbohydrate–lectin recognition events are also found to play key roles in diverse pathological processes, including infection, cancer cell metastasis, and inflammation [3]. Understanding and utilizing these interactions has therefore been the subject of active research to develop and engineer bio-analytical strategies and devices [4].

Lectin–carbohydrate interactions have been studied primarily on surfaces using bulk measurement methods including fluorescence, surface plasmon resonance (SPR) [5] and evanescent-field detection [6]. The utility of these techniques depend on how diverse libraries of ligands are immobilized on a surface and how other bio-molecules interact with them. Areas of focus—carbohydrate microarrays, have become valuable tools for high-throughput glycomic research. Different strategies have been adopted to develop array-based tools for glycomic studies including immobilizing maleimide-linked carbohydrates on thiol-derivatized glass slides [7], immobilization of oligosaccharides on nitrocellulose-coated glass slides without chemical linkers [8] and using cyclopentadiene-derivatized carbohydrates on gold surfaces [9]. Similarly, protein chips with immobilized lectins have also been used for the measurements of such interactions [4,10].

However, there is still the need to uncover the molecular basis of lectin interactions with carbohydrates and probe them at a fundamental level. The molecular recognition of lectins and carbohydrates is an intriguing process because the binding is due to relatively weak, non-covalent interactions ($K_a \approx 10^{3-4}\,M^{-1}$). However the strength and specificity required for proper cellular targeting is high [11]. Assessing protein-carbohydrate interactions has typically been difficult because of the weak affinities observed and associated complications arising from the importance of multivalency in these interactions [12].

Atomic Force Microscopy (AFM) is the ideal tool to interrogate these weak specific interactions at a molecular level. The AFM has been used as a powerful technique to image and manip-

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ulate bio-molecules under physiological conditions and at high resolution [13]. AFM based force spectroscopy has been used to investigate inter- and intra-molecular forces at the single molecule level,[14–16] and in particular, for probing biomolecular recognition events such as between complementary DNA sequences, antigen–antibody [17] and ligands and cell-surface receptors [18,19].

Concanavalin A (Con A) is a widely investigated lectin that binds specifically to α-D-mannosyl and α-D-glucosyl residues [3]. The interaction between Con A and corresponding simple sugars has been earlier studied by AFM. Lekka et al. studied the interaction force occurring between Con A and the carbohydrate component of the glycoproteins [20], Touhami et al. reported that the unbinding force between Con A and oligo-glucose saccharides to be 96 ± 55 pN [21]. Ratto et al. measured the force required to rupture a polymer tethered Con A and a similarly tethered mannopyranosylphenyl isothiocyanate bond as 47 ± 9 pN [22]. These studies have reported a wide range in force values, often with mannose-containing ligands and glycopolymers, instead of the direct lectin-sugar interaction and also did not investigate the effect of multivalency on the interaction between the lectin and the sugar. At pH < 5.5 Con A exists as a two-protomer unit while at a pH > 7 it exists as a tetramer [23,24] Understanding proximity effects and multivalent binding is key to understanding biological activity at the fundamental level [3].

Chemical force microscopy (CFM) is the general technique that uses chemical functionalization of atomic force microscopy (AFM) probes with specific functionalities that permits direct investigation of these interactions [25]. Here this approach is taken to immobilize the carbohydrate or lectin molecule to create a functionalized AFM probe. A one-step protocol is used to synthesize an amine-functionalized sugar (1-amino-1-deoxy sugar) by a novel amination reaction. The utility of a self-assembled monolayer platform to immobilize specific receptor-ligand pairs on a surface for investigation at the single molecule level is shown. The versatility of this platform allows the fabrication of either a carbohydrate or lectin surface that can be easily prepared and used for the analyses of carbohydrate–protein interactions. The mixed SAM platform not only allows covalent immobilization of an amine-containing molecule (specifically mannosylamine or the lectin concanavalin A in these experiments) but also provides an inert surface over the surrounding area that minimizes non-specific interactions and provides a homogeneous environment for binding.

The AFM is used to image and measure the specific interaction forces between Con A and mannosyl groups in liquid. The specificity of the binding interactions is confirmed via the blocking of binding sites on the Con A molecule with free mannose. By varying the environmental pH, we investigate the specific effect of multivalency on the strength of the Con A and mannose interaction. In this study, experiments were conducted at a pH value of 4.8 and 7.4 to determine the multivalent interactions involved in Con A–mannose binding and thereby determine the force required for the unbinding of a single Con A–mannose bond.

2. Experimental section

2.1. Materials and instrumentation

(1-Mercaptoundec-11-yl) hexaethylene glycol (Oligoethylene glycol (OEG) terminated thiol), HS–C_{11}–$(EG)_6$OH, and (1-mercaptonhexanedecanoic acid)-N-Hydroxy Succinimidyl ester (NHS-terminated thiol) HS–C_{15}–CO–NHS, were purchased from Asemblon Inc (Redmond, WA) and SensoPath Technologies (Bozeman, MT) respectively. Epoxy glue was purchased from Epoxy Technology (Avon, OH). Tetrahydrofuran (THF), highly purified Concanavalin A from *Canavalia ensiformis* (Jack bean), type IV, D-(+)-mannose and ammonium carbonate were purchased from Sigma–Aldrich (St. Louis, MO). Phosphate-buffered saline (PBS pH 7.4) (11.9 mM phosphates, 137 mM sodium chloride and 2.7 mM potassium chloride) and Ethanol (200-proof) were purchased from Fisher Scientific. Ultrapure water (resistivity 18.2 MΩ·cm) was obtained from a MilliQ water purification system (Millipore Scientific, MA). Gold coated PPP-CONTCSAu cantilevers from Nanosensors (Neuchatel, Switzerland) and TR800PSA cantilever from Olympus (Tokyo, Japan) were used for force measurement and imaging respectively. All force spectroscopy experiments were performed using an Asylum MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA). The NMR experiments were carried out on a Varian Mercury 300 MHz NMR spectrometer (Palo Alto, CA).

2.2. Preparation and characterization of glycosylamines of mannose

Amination of mannose was performed using the Kochetkov reaction involving the treatment of reducing sugars with ammonium carbonate to create anomeric amines (1-amino-1-deoxy sugar) [26,27]. Briefly, a solution of mannose (1%) in saturated aqueous ammonium carbonate was stirred at room temperature for 5 days at room temperature (20 °C). Solid $(NH_4)_2CO_3$ was added in fractions over the course of the reaction to ensure saturation. After the reaction, the solution was dried in vacuum for 2 days. The excess solid $(NH_4)_2CO_3$ was removed by dissolving the crude glycosylamine in warm methanol. After termination of CO_2 evolution, the methanol was slowly evaporated and the residual material dried in vacuum [26]. The freshly prepared sample was dissolved in D_2O, and further characterized by ^{1}H NMR working at 300 MHz to verify the formation of the glycosylamine.

2.3. Functionalized substrate and probe preparation

Ultraflat template-stripped gold (TSG) surfaces were prepared as previously reported [28]. Template-stripped gold surfaces were obtained by stripping the mica with tetrahydrofuran. The resulting gold surfaces were then washed several times with ethanol prior to formation of the mixed SAMs.

Mixed thiol SAMs were prepared by the incubation of the freshly prepared gold surfaces in a 5 mM mixed thiol solution in ethanol for 20 h at room temperature. The molar ratio of OEG and NHS thiol was titrated to ensure separation of attachment sites for protein on the surface [29]. After SAM formation, the surfaces were rinsed with ethanol, and incubated in a 0.01 μg mL^{-1} solution of ConA in PBS buffer for 1 h at room temperature. Gold coated cantilevers (PPP-CONTCSAu) with mixed SAM coatings were prepared in the same manner. The cantilevers were incubated with mannosylamine in PBS buffer for 1 h to obtain sugar functionalized AFM tips.

Following the incubation, the resulting surface and cantilever were washed with PBS buffer to remove any unattached protein and glycosylamine. The surfaces were then placed in a fluid cell containing 500 μL of PBS for AFM imaging and force measurements. Experiments were also performed with the lectin attached to the cantilever and the aminated sugar linked to a surface via the same chemistry and identical procedure as that described above.

2.4. AFM imaging of surfaces and force spectroscopy

Spring constants of functionalized cantilevers were measured using the thermal fluctuation method [30]. TR800PSA cantilever (spring constant ∼0.15 N m^{-1}, resonance frequency 24 kHz) were cleaned using high-intensity UV light to remove organic contamination and used for imaging the surfaces in non-contact mode. PPP-CONTCSAu cantilever (spring constant ∼0.2 N m^{-1}, resonance frequency 24 kHz) functionalized with the SAM and the sugar or