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Carbohydrate Microarrays by Click Chemistry

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Self-assembled monolayer of carbohydrates were immobilized on Si and glass substrates by Cu(I)-catalyzed Huisgen [2 + 3] cycloaddition, so-called "click chemistry". The azide terminated carbohydrates were synthesized via condensation with *p*-aminophenyl pyanosides. The formation of self-assembled monolayer was confirmed by ellipsometry and XPS. The biological abilities of the self-assembled monolayer was analyzed by lectin recognition. Lectin recognition could be analyzed in nM order.

Key Words: Carbohydarte Microarray, Click Chemistry

1. INTRODUCTION

Carbohydrates play important roles in living processes, including cell differentiation, cell adhesion, immune responses, virus infection and cancer metastasis.[1] Therefore, it is important to elucidate the molecular basis for specific protein-carbohydrate recognition events. Carbohydrate-protein interactions have been investigated by biophysical and biochemical approaches, for instance, X-ray crystallography,[2] NMR spectroscopy and mutation genesis.[4] Recent rapid progress of genomics and proteomics demands high throughput methods for fast analysis of protein-carbohydrate interactions

Microarray of biomacromolecules on solids have been fabricated for a variety of applications for the fast analysis in biology over the last decade. For instance, DNA micro-arrays and DNA chips (gene chips) are extensively used for studying many genes simultaneously.[5,6] In addition, immobilized proteins have been exploited for high-throughput studies of molecular interactions and biochemical activities, and profiling protein expression in normal and diseased states.[7] Microarray carbohydrates on solid surfaces are of great promise to enable fast screening of carbohydrate-protein interactions.[8,9,10]

One major difficulty is the immobilization of carbohydrate on the substrates. The appropriate functional groups and the generally useful chemistry for attaching carbohydrates are necessary. Since carbohydrates are multifunctional compounds, the specific and the universal reaction are desirable. In this research, we apply "click chemistry" for immobilizing the carbohydrate.[11]

"Click chemistry" is the reaction of Cu(I)-catalyzed Huisgen [2 + 3] cycloaddition, which has attracted to its convenient, quick, and quantitative reaction.[12-14] This chemoselective coupling is used for various applications, including chemical modifications of polysaccharides.[15] Such applications encouraged us to establish "click chemistry for carbohydrate microarray", namely, chemoselective coupling between alkyne-immobilized substrate and azide terminated carbohydrate. The immobilization of carbohydrate was examined with several kinds of carbohydrates and the biological abilities were investigated with lectin and cell recognition.

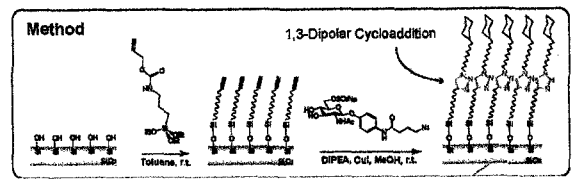

Fig.1 Schematic illustration of carbohydrate microarray by immobilization of click chemistry.

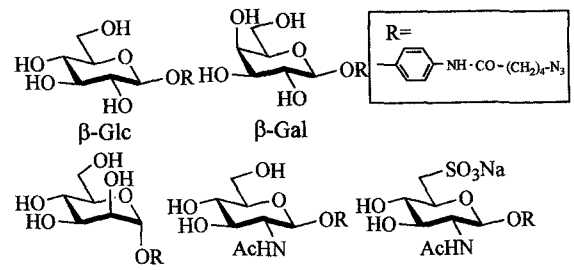

Fig. 2 Molecular Structure used in the research.

2. EXPERIMENTAL

2.1 Syntheses

Azide terminated carbohydrates (Fig. 2) were synthesized by a condensation of azide terminated carboxylic acid and *p*-aminophenyl pyranoside. The *p*-aminophenyl pyranossides were synthesized via a previous methods. The carbohydrates were

characterized by ^{1}H-NMR (Varian Inova 500). The purities were confirmed by HPLC and TLC with two different solvent systems.

2.2 Measurements of substrates[16]

The static contact angle of water on the prepared surfaces was measured at 25 °C by operating a drop shape analysis system of DSA 10 Mr2 (Kruss, Germany). The thickness of the film on Si(100) substrate was measured with a PZ2000 ellipsometer (Philips, Holland) using a He-Ne laser of 632.8 nm, in which the incident angle was 70° from the normal. The FTIR spectra in the transmission method under a N_2 atmosphere were recorded with an FTIR 7000 (Digilab Laboratories, USA) with 1000 times of interferogram accumulation. XPS analysis was performed using an ESCA-3300 (Shimadzu/Kratos, Kyoto, Japan) with a collection angle of 45° from the normal. Fluorescence microscopic studies were performed using a Zeiss Axiover 200 M laser confocal microscope (Carl Zeiss Inc., Germany) equipped with external argon laser (for excitation at 490 nm).

2.3 Carbohydarte Immobilization

The Si or glass substrate was washed by acetone, ethanol and milliQ water with sonication for 10 min. Then the substrate was cleaned by UV/O_3 irradiation. The substrate was immersed in 1 mM toluene solution of *O*-(propagyl)-*N*-(trimethoxysilyl propyl)urethane under N_2. The substrate with self-assembled monolayer was rinsed with toluene, ethanol and milliQ waterwith sonication for 2 min. Then, the substrate was immersed with azide terminated carbohydrate solution with DIEA and Cu(I) in MeOH. After incubation with carbohydrates, the substrate was washed with ethanol and millQ water.

2.4 Lectin recognition

To evaluate the lectin recognition ability, the saccharides immobilized substrate was incubated with FITC-labeled lectins. The substrate was immersed lectin solution in PBS buffer for 1 h, and the substrate was rinsed with PBS buffer. The remained lectin on the substrate was observed by fluorescence microscopy and the fluorescence image was recorded with a fluorescence microscope and analyzed with Scion image software (version 4.02 beta, Scion Co., Frederick, MD).

2.5 Cell culture

NIH 3T3 fibroblasts were incubated in DMEM supplemented with 10% FCS, 1% NEAA, 1% NaPyr, 100 mU/mL of penicillin, and streptomycin. NIH 3T3 cell were plated on the carbohydrate-immobilized substrate at a concentration of. 3.0×10^3 cell/ml. The substrates were incubated for 6, 24, and 72 h in 10 % FBS DMEM, and then rinsed with PBS to remove non-adherent cells. The cell number was assayed with WST-8.

3. RESULTS and DISCUSSION

3.1 Self-Assembled monolayer of Alkyne-terminated organoslilane.

Click chemistry is related to Huisgen [2 + 3] cycloaddition with azide and alkyne. The azide terminated carbohydrates were used in this research, and the substrate was treated with alkyne terminated organosilane of *O*-(propagyl)-*N*-(trimethoxysilyl propyl)urethane. The substrate was immersed in toluene solution to form the self-assembled monolayer (SAM) and thickness was measured by ellipsometery (Fig. 3) . The thickness of SAM gradually increased, and the saturated thickness was about 11 Å, which is suitable to the molecular dimension of the corresponding organosilane. The formation of SAM was also confirmed by contact angle, FTIR, and XPS measurements; The contact angle changed from 0° to 77°. Typical bands due to the organosilane was observed in FTIR (3100-3600cm^{-1} alkyne and urethane, 2900cm^{-1} alkyl, 1100cm-1 ethoxy silyl) and XPS (C-C peak around 285 eV).

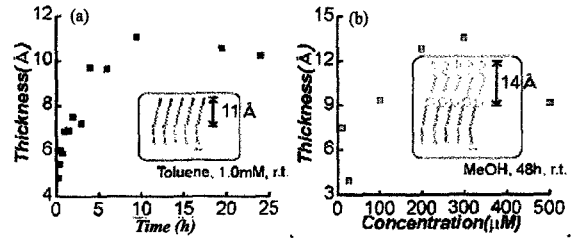

Fig. 3 Immobilization of Carbohydrate. Thickiness of (a) *O*-(propagyl)-*N*-(trimethoxysilyl propyl)urethane and (b) azide terminated β-GlcNAc.

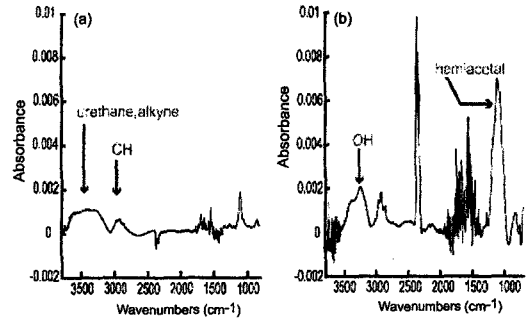

Fig. 4 FTIR transmittance spectra of the Si substrates. (a) alkyne terminated SAM and (b) β-GlcNAc immobilized substrate.

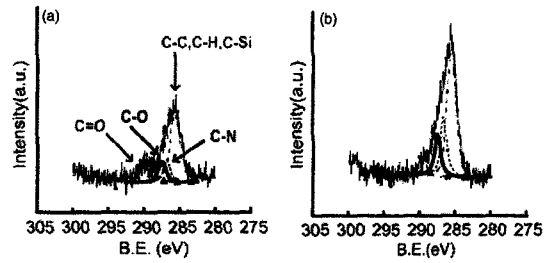

Fig. 5 XPS of the Si substrates. (a) alkyne terminated SAM and (b) β-GlcNAc immobilized substrate.

3.2 Immobilization of cabohydartes on subtstrates

The resultant alkyne terminated substrate was subjected to cycloaddition reaction with azide terminated carbohydrates using Cu(I) and DIEA. The reaction readily proceeded in both methanol and DMSO solution, shown by the thickness by ellipsometery. The saturated thickness of carbohydrate was 14Å that was

accoding to the MM calculation. The formation of SAM was also confirmed by contact angle, FTIR, and XPS measurements; The contact angle was decreased from 77° to 60° due to the sugar moiety. Typical bands due to the organosilane were observed in FTIR (3100-3500cm^{-1} νO-H, 1000-1200cm^{-1} δC-O-C hemiacetal of carbohydrate). The C1s spectra in XPS showed the shoulder peak around 280-290 (eV), which indicate the C-O of saccharides and esters and C=O of esters and amide of the azide terminated carbohydrates. These results indicated that carbohydrate was densely immobilized by cycloaddition. In addition, the density of carbohydrate could be adjusted by change of reaction time.

There are several methods to immobilize the carbohydrates. One major method is the utilization of artificial glycolipid with thiol or disulfide for immobilization via Au-S bond.[17] However, the artificial glyolipids are difficult to immobilize in a densely packed manner due to the amphiphilicity in the aqueous solution. On the other hand, the clicking immobilization of azide carbohydrates can be applied to many kinds of saccharide and easy to control the self-assembling property.

3.3 Lectin recognition

The protein recognition of the carbohydrate-immobilized substrate was examined using lectins (RCA_{120} and WGA) and other proteins (BSA and b-FGF)(Fig. 6). The substrate with five different carbohydrate (β-Gal, β-Glc, α-Man, β-GlcNAc, and 6-Sulfo-β-GlcNAc) was incubated with FITC-labeled proteins. The fluorescence was observed to the specific carbohydrate. That is, RCA_{120} (β-Gal recognition lectin) attached to β-Gal. WGA (β-GlcNAc recognition lectin) attached to β-GlcNAc and 6-sulfo-β-GlcNAc. The non-specific adsorption to other carbohydrate and to glass substrate was scarcely observed, and the signal to noise ration was about 5. The fluorescence of the lectin can be observed from 5 nM. Adsorption of other protein was also analyzed. Adsorption of BSA to saccharide was less than 20 % that of the appropriate lectin. Adsorption of bFGF also didn't show specificity. The bFGF bound to 6-sulfo-GlcNAc most, but the amount of bound to carbohydrate was in the same order.

These results showed that the carbohydrate-immobilized substrate was suitable to analyze the carbohydrate-protein interaction. Lectin recognition of the substrate showed enough specificity, and the interaction with other proteins was also to be analyzed.

3.4 Cell culture on the carbohydrates

The cell adhesion and the cell viability were examined on the carbohydrate-immobilized substrates (Fig.7). After seeding NIH 3T3, the number of cells on the substrate was assayed with WST-8. The number of the cell on the carbohydrates were more than that of glass substrate. Initially the cell adhesion preferentially occurred on 6-sulfo-GlcNAc, but after 72 h incubation the cell adhesion was more on the Glc immobilized substrates.

The cell adhesion on 6-sulfo-GlcNAc might occur in a similar way to proteoglycan. For example, heparin adsorbed and form supramolecules of β-FGF, which induce the cell adhesion via interaction with bFGF.

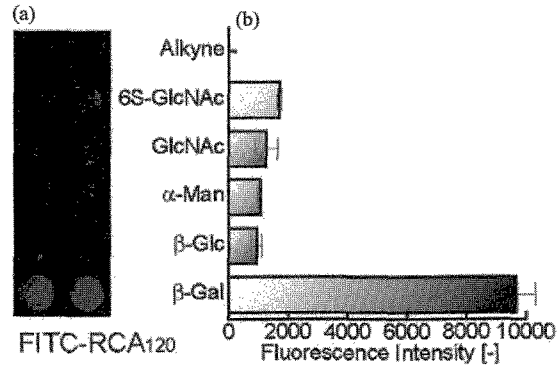

Fig. 6 Lectin recognition. (a) fluorescent image of FITC-RCA_{120}, and (b) fluorescence intensity on carbohydrate microarray.

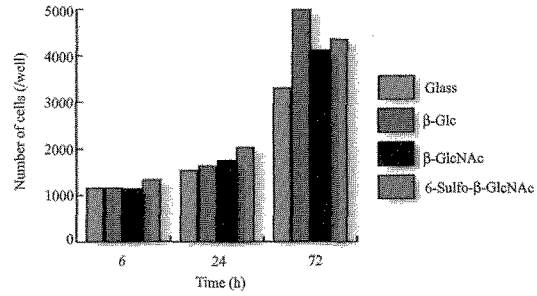

Fig. 7 Cell adhesion on carbohydrate microarray.

However, the densely packed sulfated group prevent the protein non-specific adsorption and adhesion of phospholipids. Therefore, β-Glc immobilized substrate showed better biological abilities.

4. CONCLUSION

We have demonstrated that carbohydrates can be immobilized by Cu(I)-catalyzed Huisgen [2 + 3] cycloaddition. It was possible to obtain the densely packed glyco-monolayer. The carbohydrate-immobilized substrate showed specific and strong lectin recognition, and the cell attachment to the substrate also can be analyzed. The immobilized of carbohydrate via click chemistry is easy and useful method to fabricate the carbohydrate micro array.

Acknowledgment

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