Carbohydrate Polymers 92 (2013) 374–379

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Hybrid immobilization of galactosyl lactose and cellobiose on a gold substrate to modulate biological responses

Takuya Kitaoka*, Chiharu Yoshiyama, Fumi Uemura

Department of Agro-Environmental Sciences, Graduate School of Bioresource and Bioenvironmental Sciences, and Biotron Application Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

ARTICLE INFO

Article history:
Received 7 September 2012
Received in revised form
27 September 2012
Accepted 28 September 2012
Available online 5 October 2012

Keywords:
Biointerface
Cell culture scaffold
Cytochrome P450 activity
Glycomaterial
Lectin-binding assay
Self-assembled monolayer

ABSTRACT

Bioactive *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose (4′-galactosyl lactose) was site-selectively modified at a reducing end with thiosemicarbazide (TSC). As-synthesized 4′-galactosyl lactose-TSC was immobilized on a gold substrate with cellobiose-TSC as a spacer through spontaneous self-assembly chemisorption *via* S—Au bonding. Quartz crystal microbalance analysis suggested the successful formation of self-assembled monolayers (SAMs) of 4′-galactosyl lactose-TSC and/or cellobiose-TSC. Galactose-binding lectin exhibited the highest affinity for hybrid SAMs with an equimolar ratio of the two oligosaccharide-TSCs, while glucose-binding lectin showed decreasing adsorption with a decrease in cellobiose-TSC ratios. Human hepatocellular carcinoma cells, which recognize galactose residues, efficiently adhered to the hybrid SAMs. Higher enzymatic deethoxylation of ethoxyresorufin *via* cytochrome P450 appeared on hybrid SAMs. These results suggested that clustering of the bioactive sugars was involved in the cellular responses, possibly *via* biological carbohydrate–protein interactions. This approach to designing carbohydrate-based scaffolds should provide a basis for the functional development of glyco-decorated biointerfaces for cell culture applications.

1. Introduction

Carbohydrates are abundantly expressed on the outer surfaces of cytoplasmic membranes of mammals as well as viral, bacterial, protozoan and fungal pathogens (Hayashi et al., 2004). Biological regulation of cell attachment and growth is mostly governed by carbohydrate-mediated communications at cellular interfaces *via* protein–carbohydrate (nonreducing ends) interactions (Hu et al., 2010; Wang, Yin, Wang, & Wang, 2012; Wu, Yao, Bai, Du, & Ma, 2010). For tissue engineering applications, innovative cell culture scaffolds that possess various biological functions for cellular activation and organization are required (Kumar et al., 2011; Peter et al., 2010; Tsai, Chen, Li, Lai, & Liu, 2012). Thus, carbohydrate-based scaffolds for cell culture applications may be useful for advancing cell engineering technology.

Lactose, *O*-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose, is a major disaccharide found notably in mammalian milk, and can mediate various biological phenomena *in vivo* (Bajdik et al., 2009; Miyagawa, Carmelita, Kasuya, & Hatanaka, 2007; Potes, Kerry, & Roos, 2012). Recently, the galactose residues of lactose and its oligomers have attracted attention as functional bioregulators for *in vitro* cell culture applications. Especially, it is well known that various carbohydrate-binding proteins, called a lectin, on cell membranes have a broad range of interfacial biofunctions including cell–cell adhesion and proliferation (Vasta & Ahmed, 2008). On these grounds, Akaike et al. reported that poly-*N*-*p*-vinylbenzyl-D-lactoneamide (PVLA) was useful as a hepatocyte culture matrix by noting that asialoglycoprotein receptors, one of the calcium-dependent (C-type) lectins, on rat liver cells can interact with the galactose branch of the PVLA backbone (Uchida, Serizawa, Ise, Akaike, & Akashi, 2001). Gotoh, Niimi, Hayakawa, and Miyashita (2004) reported the hepatocyte culture behavior of lactose-immobilized silk fibroins and their resulting galactose-mediated biological functions. Thus, continued research in glycomaterial engineering should help in the development of practical cell culture scaffolds.

In our previous studies, various types of oligo-/polysaccharides such as cellulose and its derivatives (Kitaoka, Yokota, Opietnik, & Rosenau, 2011; Yokota, Kitaoka, Opietnik, Rosenau, & Wariishi, 2008; Yokota, Kitaoka, Sugiyama, & Wariishi, 2007; Yokota, Kitaoka, & Wariishi, 2008), chitin (Yoshiike & Kitaoka, 2011; Yoshiike, Yokota, Tanaka, Kitaoka, & Wariishi, 2010), chitosan (Yoshiike et al., 2010) and hyaluronan (Tanaka, Yoshiike, Yoshiyama, & Kitaoka, 2010) were immobilized on a gold substrate through a

* Corresponding author. Tel.: +81 92 642 2993; fax: +81 92 642 2993.
E-mail address: tkitaoka@agr.kyushu-u.ac.jp (T. Kitaoka).

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2012.09.088

self-assembly chemisorption technique, and some were tested in cell culture applications (Tanaka et al., 2010; Yokota, Kitaoka, & Wariishi, 2008; Yoshiike & Kitaoka, 2011; Yoshiike et al., 2010). Only reducing end groups of carbohydrates were selectively modified with thiosemicarbazide (TSC). The resultant carbohydrate-TSC derivatives were fixed on the gold (Au) surfaces *via* a covalent S—Au bond, called vectorial chain immobilization. This approach has provided unique biointerfaces containing aligned carbohydrates whose bioactive nonreducing ends are in direct contact with the surfaces of cultured cells. Rat liver cells (IAR-20) (Yokota, Kitaoka, & Wariishi, 2008), human hepatocellular carcinoma cells (HepG2) (Yoshiike & Kitaoka, 2011) and mouse fibroblasts (NIH-3T3) (Tanaka et al., 2010; Yoshiike et al., 2010) were preferentially adhered to these biointerfaces. In particular, the combination of chitin and cellulose having high affinity for each other at the molecular level (Takegawa, Murakami, Kaneko, & Kadokawa, 2010) demonstrated the unique biological response of HepG2 cells on the hybrid nanolayers prepared using respective chito-/cello-hexaose (Yoshiike & Kitaoka, 2011). Further expansion of this technique is expected to be applicable for the functional design of bioactive glyco-scaffolds.

In this study, bioactive *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose (4′-galactosyl lactose; GL) and cellobiose (CL) as a spacer were site-selectively modified at each reducing end group with TSC. The hybrid glyco-nanolayers were fabricated in various molar ratios of both oligosaccharides according to our previous study (Yoshiike & Kitaoka, 2011). Herein, the combination of longer-chain bioactive GL (trisaccharide) and shorter-chain spacer CL (disaccharide) was applied to expect higher bioactivity due to the presented galactose residues on the scaffold surfaces. The self-assembly behavior of carbohydrate-TSCs was monitored by quartz crystal microbalance (QCM) analysis, and the as-formed self-assembled monolayers (SAMs) were characterized by a lectin-binding assay on a QCM apparatus to assess the essential interaction between as-designed glyco-SAMs and carbohydrate-binding proteins at the molecular level. We evaluated the hydrophilic properties of carbohydrate-SAMs by measuring the sessile-droplet contact angle of water. HepG2 cells, which contain galactose receptors, were used to investigate the biofunctional properties of the GL/CL hybrid nanolayers by examining their initial adhesion and their cytochrome P450 activity.

2. Experimental

2.1. Materials

Pure *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose (GL), resorufin, resorufinethylether and 3-methylcholanthrene were purchased from Wako Pure Chemical Ind. Ltd., Japan. Cellobiose (CL), thiosemicarbazide (TSC), sodium cyanoborohydride ($NaCNBH_3$) and dicumarol were purchased from Sigma–Aldrich Corp., USA. Microcover glass (diameter: 15 mm, Matsunami Glass Ind. Ltd., Japan) was used as a transparent substrate for thin-layer gold sputtering. Water was purified with a Milli-Q system (Millipore Co. Ltd., Japan). Human liver carcinoma cells (HepG2) were provided by the Japanese Collection of Research Bioresources, Japan. Dulbecco's Modified Eagle's Medium (DMEM), glutamine, penicillin–streptomycin, trypsin–EDTA and MEM non-essential amino acids solution (NEAA) were purchased from Life Technologies Corp., Japan. Fetal bovine serum (FBS) was obtained from Biowest Co. Ltd., France. Tissue culture polystyrene (TCPS) dishes and TCPS plates (24 well) were obtained from Sumitomo Bakelite Co. Ltd., Japan. Concanavalin A (ConA from *Canavalia ensiformis*, Wako Pure Chemical Ind. Ltd., Japan) and *Ricinus communis* agglutinin (RCA_{120}, Cosmo Bio Co. Ltd., Japan) were used for the lectin-binding assay to assess the biological recognition of glucose and galactose residues, respectively, on the SAM surfaces. Other chemicals were reagent grade and used without further purification.

2.2. Preparation of GL/CL hybrid nanolayers

An outline of the preparation of hybrid glyco-nanolayers composed of GL and CL is illustrated in Fig. 1. Terminal TSC-labeling of GL and CL molecules at each reducing end was successfully carried out by aqueous reductive amination with $NaCNBH_3$ under mild conditions as previously reported (Tanaka et al., 2010; Yoshiike & Kitaoka, 2011; Yoshiike et al., 2010). Either powdery GL (20.0 mM final concentration) or CL (29.2 mM) was dissolved in Milli-Q water, and then the TSC reagent (110 mM) was added to the solutions, followed by stirring at 70 °C for 72 h in the presence of $NaCNBH_3$ (2 M). As-synthesized GL-TSC and CL-TSC as shown in Fig. 1 were precipitated in ethanol, and rinsed using sufficient ethanol by repeated (at least five times) centrifugation (3000 rpm, room temperature) to remove excess $NaCNBH_3$ and residual TSC.

Piranha-washed clean glass plates were coated with Au by ion sputtering (VPS-020, ULVAC Inc., Japan) at 4 mA current for 3 min below 1.5 mPa of vacuum level. The Au layer thickness was *ca.* 23 nm (Yoshiike & Kitaoka, 2011). The Au-coated plates were soaked at room temperature for 24 h in the designated concentration of carbohydrate-TSC aqueous solutions containing GL-TSC and CL-TSC at molar ratios of 1:0, 3:1, 1:1, 1:3 and 0:1. The treated plates were then washed with sufficient Milli-Q water at least three times to give GL/CL hybrid nanolayers (pure GL-SAM; nominally 75% GL-SAM, 50% GL-SAM and 25% GL-SAM; and pure CL-SAM).

2.3. Analytical characterization

A QCM apparatus (AFFINIXQ, Initium Inc., Japan) equipped with a 27 MHz AT-cut, Au-coated quartz crystal was used to quantify the amounts of carbohydrate-TSC molecules chemisorbed on the Au surface of the QCM sensor chip. An aqueous solution of carbohydrate-TSCs (1.0 mM, 15 μL, GL-TSC:CL-TSC = 1:0, 3:1, 1:1, 1:3 or 0:1 molar ratio) was injected into a sample chamber containing 8.0 mL of Milli-Q water at pH 7 for 24 h. The frequency changes of the sensor chip were monitored on line at 25 °C with stirring at 1000 rpm. The approximate amounts of chemisorbed oligosaccharides were calculated according to our previous study (Yoshiike & Kitaoka, 2011).

Subsequently, the adsorption behavior of the lectins onto the carbohydrate-SAMs was monitored by QCM analysis. Each carbohydrate-SAM was set into a sample chamber containing 8.0 mL of phosphate buffered saline (PBS) solution with calcium chloride dehydrate and magnesium chloride hexahydrate at pH 7 and 25 °C with stirring at 1000 rpm. After adding 30 μL of a blocking agent (4× Blocking Reagent for AFFINIXQ, Initium Inc., Japan), the excess amount of the blocking agent was sufficiently removed by rinsing with PBS solution. After stabilization of the frequency variation, either ConA or RCA_{120} (1.0 μM, 8.0 μL) was added four times at an interval of 30 min during the 2 h monitoring of continuous frequency changes.

2.4. Cell culture assay

Hybrid nanolayers on Au-coated glass plates were placed at the bottom of each well of 24-well TCPS plates, and suspended HepG2 cells (1.0 mL) were seeded on each substrate (1.0×10^5 cells mL^{-1}, *i.e.* 1.0×10^5 cells per well). After incubation for 24 and 96 h with DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin–streptomycin and 1% (v/v) NEAA in a 5% CO_2 atmosphere at 37 °C, unattached cells were completely removed