purified silicateins then were reconstituted to their native form by dialysis (at 2 °C) from buffered guanidinium hydrochloride and mercaptoethanol to permit proper refolding and formation of intramolecular disulfide bonds. The reconstituted proteins then were immediately adjusted to equivalent protein concentrations and assayed in triplicate for their catalysis of silica synthesis from tetraethoxysilane at neutral pH (1 h, 20 °C) under conditions described previously.[3] The silica product was washed by centrifugation, dried by evaporation and quantified colorimetrically with the molybdate reagent after hydrolysis with alkali. Results are expressed as relative specific activities of alkoxysilane polycondensation after correction for the yield of the uncatalyzed reaction. Under these conditions, the average specific activity of the native reconstituted silicatein α protein was 140.0 ± 6.2 nmol silica synthesized per hour per 60 μg protein; the control value obtained in the absence of any protein was 6.7 ± 2.1 nmol silica synthesized per hour.

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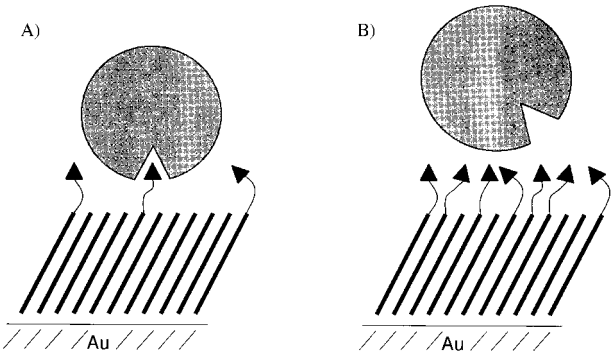
The Role of Ligand Density in the Enzymatic Glycosylation of Carbohydrates Presented on Self-Assembled Monolayers of Alkanethiolates on Gold**

Benjamin T. Houseman and Milan Mrksich*

The enzymatic modification of immobilized carbohydrates is important in protein trafficking,[1] viral and bacterial pathogenesis,[2] and cell migration.[3] Many model systems, including those that use polymers,[4] dendrimers,[5] and liposomes[6] to present carbohydrates, have provided information on the interactions between proteins and immobilized sugars. These systems, however, present carbohydrates in a heterogeneous environment and offer little flexibility in tailoring the structure of groups surrounding the ligands. These limitations make mechanistic studies of enzymatic processes at interfaces difficult.

Self-assembled monolayers (SAMs) of alkanethiolates on gold are structurally well defined substrates that represent an excellent model system for studies in bio-interfacial science.[7] Several reports have utilized SAMs to examine the interactions between immobilized ligands and proteins in solution. It is clear from much of this work that the accessibility of a ligand will influence the ability of a protein to bind it.[8a–d] Ligands immobilized at higher densities,[8e–f] for example, may have biological properties that are substantially different from those of the same ligand presented at a low density (Scheme 1). Here we report the use of mixed SAMs that present *N*-acetylglucosamine (GlcNAc) and tri(ethylene



Scheme 1. Ligands immobilized at low density (A) should be more accessible for binding to protein than are ligands immobilized at high density (B).

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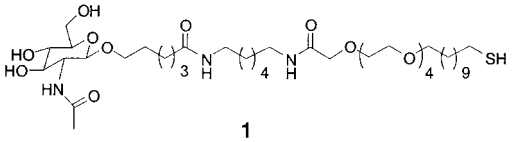
glycol) groups to demonstrate that the enzymatic activity of bovine β-1,4-galactosyltransferase (GalTase) depends on the density of immobilized carbohydrate.

We prepared substrates from alkanethiol **1**, terminated in a GlcNAc group, and a second alkanethiol **2**, terminated in the tri(ethylene glycol) group.[9, 10] The carbohydrate is a substrate



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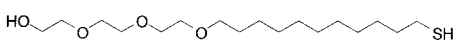

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for GalTase, while the tri(ethylene glycol) groups resist the nonspecific adsorption of protein to the model substrate.[11] The density of GlcNAc at the interface is easily controlled by adjusting the ratio of alkanethiols in the solution from which the monolayer forms. In the presence of divalent manganese ions, GalTase transfers galactose from the donor substrate, uridine diphosphogalactose (UDP-Gal), to the 4-hydroxyl group of GlcNAc (Figure 1).[12] To quantitate the transfer of galactose to the immobilized GlcNAc groups, buffer containing enzyme, [^{14}C]UDP-Gal, UDP-Gal, and $MnCl_2$ was placed onto monolayers and kept for eight hours at 37 °C. The substrates were then placed in aqueous solution, and the alkanethiolates were removed from the gold surface by UV irradiation. The supernatant was analyzed with liquid scintillation counting. This protocol was repeated for monolayers presenting different densities of GlcNAc.

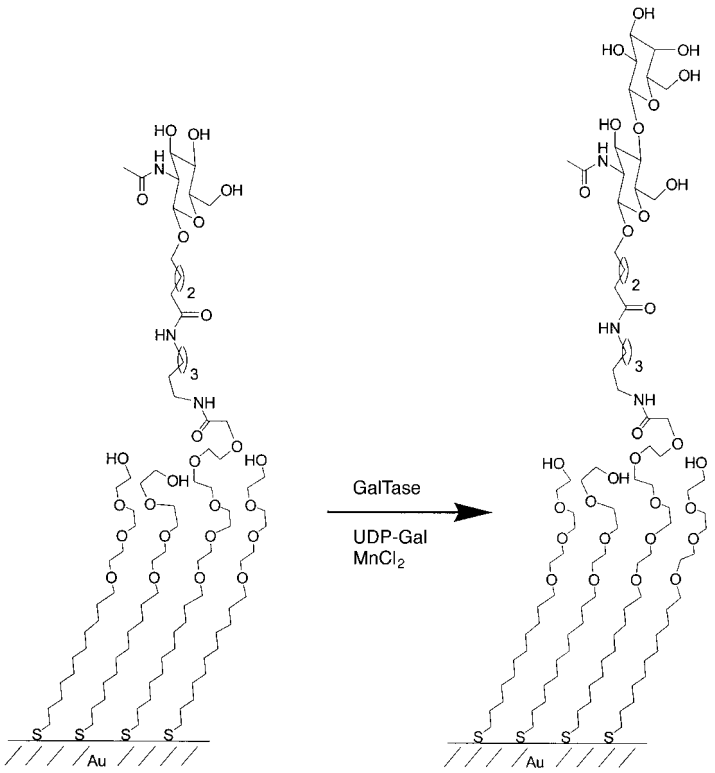


Figure 1. This work used monolayers presenting the carbohydrate *N*-acetylglucosamine mixed with tri(ethylene glycol) groups as a model substrate to determine the relationship between GalTase-mediated glycosylation of the sugar and the density of carbohydrate on the monolayer.

Figure 2 shows the relationship between the density χ_{GlcNAc}[13] of GlcNAc on the monolayer and the incorporation of ^{14}C-labeled galactose. Incorporation of radionuclide increased linearly with density of GlcNAc until $\chi_{GlcNAc} = 0.7$,

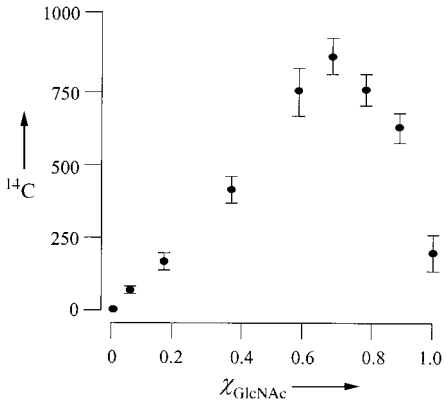
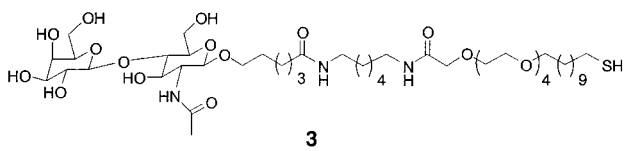


Figure 2. Relationship between χ_{GlcNAc} and the enzymatic incorporation of ^{14}C from radiolabeled UDP-Gal. A solution (40 μL) containing GalTase (25 mU), $MnCl_2$ (10 mM), UDP-Gal (0.4 nmol), and [^{14}C]UDP-Gal (0.2 nmol) in HEPES buffer (50 mM, pH 7.5) was applied to monolayers having values of χ_{GlcNAc} ranging from 0 to 1.0. The reactions were allowed to proceed for eight hours at 37 °C before the coverslips were rinsed with distilled water, dried under a stream of nitrogen, and placed in scintillation vials containing 750 μL of distilled water. The monolayers were desorbed by exposure to UV light for 4×15 min, and the amount of ^{14}C incorporation was measured with a liquid scintillation counter. Incorporation of radionuclide was normalized to the area of monolayer (values are reported in units of counts per cm^2). Each data point represents the average of four to eight samples.

where it reached a maximum, and then decreased with larger values of χ_{GlcNAc}. Monolayers that presented carbohydrate alone ($\chi_{GlcNAc} = 1.0$) showed an extreme reduction in glycosylation; the amount of [^{14}C]galactose on these monolayers was equal to that observed on monolayers with $\chi_{GlcNAc} = 0.2$. The low yield was not due to a slower rate of reaction since no further glycosylation was observed after the incubation of eight hours. These substrates did not suffer from nonspecific incorporation, since only background levels of [^{14}C]galactose were detected on monolayers presenting tri(ethylene glycol) groups alone. To illustrate the specificity of the reaction, we subjected monolayers prepared from **2** and the alkanethiol **3**, terminated in a *N*-acetyllactosamine (LacNAc) group, to the reaction conditions and observed only background levels of



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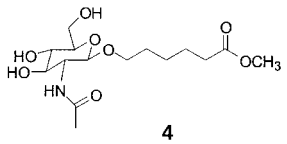

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^{14}C incorporation. These data show clearly that steric crowding of carbohydrate ligands at the interface inhibits enzyme–substrate interactions in the model system described here; the effect became evident when χ_{GlcNAc} was greater than 0.7. It was not possible to determine whether the decrease in glycosylation resulted from crowding of unmodified ligands or from the influence of LacNAc groups present on a partially modified substrate.

We used surface plasmon resonance (SPR) spectroscopy to measure the association of two lectins having specificity for either GlcNAc or LacNAc with the monolayers. The *BS-II* lectin from *Bandeiraea simplicifolia* binds terminal, nonreducing GlcNAc groups ($K_d \approx 80$ nM),[14] while the lectin from *Erythrina cristagalli* binds terminal LacNAc groups ($K_d \approx 400$ μM).[15] SPR spectroscopy is an optical technique that measures the angle θ at which polarized light, reflected from the back of a gold-coated slide supporting a SAM, shows a minimum in intensity. The magnitude of this angle depends linearly on the change in refractive index—and therefore the mass of the protein—in the interfacial region.[16, 17] The specific interaction of the lectins with the monolayers determines the amount of each carbohydrate present on the substrate.[18] The high molecular weight of the lectins[19] is beneficial as it provides a larger SPR response, and hence a more sensitive measurement, for the binding event.

Figure 3A shows the SPR response curves for the binding of each lectin to a monolayer presenting GlcNAc ($\chi = 0.01$). The *B. simplicifolia* lectin (1 μM in PBS) bound carbohydrate groups at the surface. When the protein solution was replaced with buffer, the amount of immobilized protein fell slowly. Since the lectin can bind cooperatively,[19] much of the protein remained associated with the substrate. The addition of GlcNAc derivative **4** (1 mM in PBS) and subsequent treatment with buffer removed nearly all of the lectin from the monolayer.[20] The lectin from *E. cristagalli* (5 μM in PBS), by contrast, showed no association with GlcNAc groups on the monolayer.[21]

The response curves in Figure 3B show the association of each lectin with a monolayer presenting LacNAc groups ($\chi = 0.01$). As expected, the *E. cristagalli* lectin, but not the *BS-II* lectin, bound the disaccharide at the interface. The lectin dissociated upon introduction of soluble LacNAc (2 mM in PBS) and treatment with buffer.[21] These experiments demonstrate that each lectin exhibits absolute specificity in binding its ligand.

We used SPR spectrscopy to characterize the yield of galactosylation for the enzymatic reaction. A monolayer presenting GlcNAc ($\chi = 0.01$) was treated for 15 min with buffer containing GalTase, $MnCl_2$, and UDP-Gal. The solution was replaced with running buffer, and a solution of each lectin was introduced for 20 min to bind GlcNAc or LacNAc groups on the monolayer. Figure 4A shows that the *E. cristagalli* lectin, but not the *BS-II* lectin, associated with the monolayer and provides clear evidence that the yield of this transformation was essentially quantitative. To characterize the reaction further and to demonstrate that this approach could provide kinetic information, we used the *BS-II* lectin to examine glycosylation after exposure to the enzyme for different amounts of time. A lower concentration of enzyme

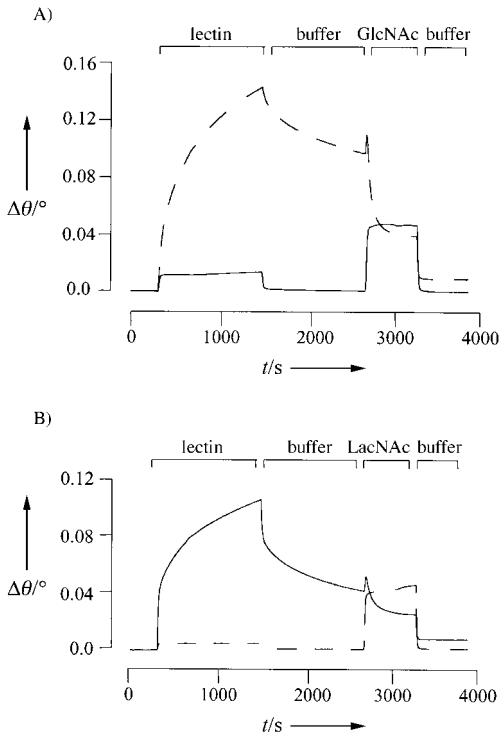

Figure 3. Data from SPR investigations for the binding of *Bandeiraea simplicifolia BS-II* lectin (1 μM in PBS, dashed curve) and *Erythrina cristagalli* lectin (5 μM in PBS, solid curve) to monolayers presenting A) 1% GlcNAc or B) 1% LacNAc among tri(ethylene glycol) groups. A solution of lectin in PBS was allowed to flow over the monolayers for 20 min, replaced with buffer for 20 min, and followed by a solution of soluble carbohydrate ligand (1 mM **4** or 2 mM LacNAc in PBS) for 10 min. The *BS-II* lectin bound to monolayers presenting GlcNAc groups, while the *E. cristagalli* lectin bound only to monolayers presenting LacNAc moieties. Binding of both lectins could be competitively inhibited by the introduction of soluble carbohydrate ligand.

was used in these experiments to slow the rate of reaction and permit observation by SPR spectroscopy. Figure 4B shows that a time-dependent decrease in the binding of the *B. simplicifolia* lectin occurs. After 30 min the binding of lectin decreased to background levels because all GlcNAc groups had been modified by the enzyme. No decrease in lectin binding was observed when either $MnCl_2$ or UDP-Gal was omitted from the reaction mixture. These data show how the combination of SPR spectroscopy and carbohydrate-specific lectins can be used to provide kinetic information for enzymatic modification of immobilized substrates.

This work provides a clear demonstration of the extent to which the density of an immobilized ligand can affect its biological properties. This effect must be considered in model systems that present biologically active ligands at high density. This report also highlights several characteristics of SAMs that make them the best available class of model substrates for studies in bio-interfacial science: They are inert to the nonspecific adsorption of protein; the density of ligand at the interface can be controlled; SPR spectroscopy can be used to measure the association of proteins with immobilized ligands; and the structure and environment of ligands at the interface can be tailored through synthetic organic chemistry. The recognition and glycosylation of immobilized GlcNAc by GalTase has a special relevance to bio-interfacial phenomena,

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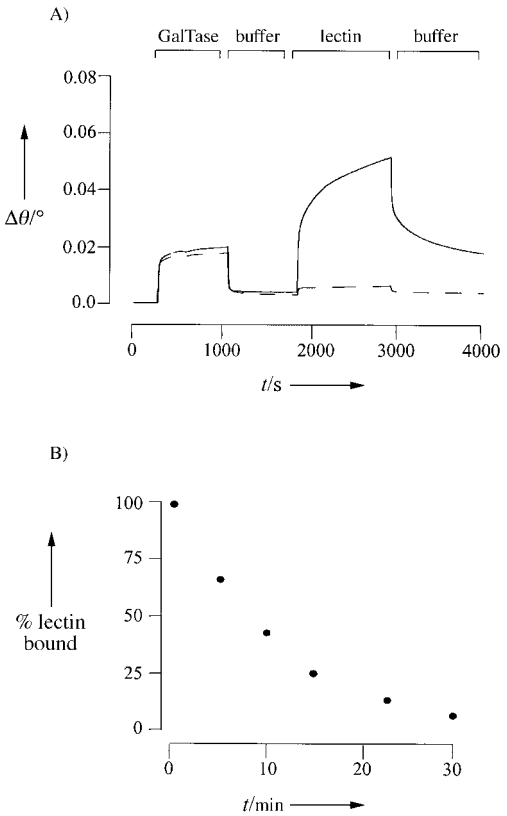

Figure 4. A) The *E. cristagalli* lectin, but not the *BS-II* lectin, binds LacNAc groups resulting from glycosylation of immobilized GlcNAc by GalTase. Monolayers were mounted in the SPR flow cell and treated with buffer containing GalTase (15 μM in PBS), UDP-Gal (0.4 nmol), and $MnCl_2$ (10 mM) for 15 min. The reaction mixture was replaced with running buffer for 15 min, and a solution of each lectin (*E. cristagalli* = solid curve; *BS-II* = dashed curve) was introduced to determine the presence of GlcNAc or LacNAc on the monolayer. Quantitative conversion of GlcNAc groups into LacNAc groups was observed. B) Plot of *BS-II* lectin binding versus time. Separate monolayers were treated with the enzyme cocktail (1.75 μM GalTase) in the SPR flow cell for different amounts of time, and binding of the lectin to the modified surfaces was recorded. All data are reported as a percentage of the binding response observed between the lectin and an untreated monolayer presenting GlcNAc ($\chi = 0.01$).

as it is a key determinant of the migration of cells on laminin matrices.[3a] We believe that the model system described here will be important for elucidating mechanistic details of this migration process.

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[10] Substrates were prepared by the evaporation of titanium (1 nm) and then gold (12 or 38 nm) onto glass coverslips. The slides were cut into pieces approximately 1 cm^2 in size and immersed in an ethanolic solution (0.4 mL) containing different ratios of conjugate **1** or **3** and alkanethiol **2** (total concentration of thiol 1 mM). After eight hours the substrates were removed from the solutions, rinsed with absolute ethanol, and dried under a stream of nitrogen.

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[18] All substrates, including those that presented GlcNAc or LacNAc alone, resisted the nonspecific adsorption of even the "sticky" protein fibrinogen (1 mg mL^{-1} in PBS).

[19] The *BS-II* lectin is a tetramer of four identical subunits (112 kDa), while the lectin from *Erythrina cristagalli* is a homodimer (57 kDa).

[20] The small degree of irreversible binding present after the replacement of soluble carbohydrate with running buffer probably resulted from the nonspecific adsorption of protein to defect sites in the monolayer.

[21] The changes in $\Delta\theta$ shown in the figure result from an increase in the refractive index of the lectin solution and the carbohydrate solution relative to the buffer; the same changes in $\Delta\theta$ were observed on surfaces presenting tri(ethylene glycol) groups alone.

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