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Conformationally Constrained Functional Peptide Monolayers for the Controlled Display of Bioactive Carbohydrate Ligands

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Abstract

In this study we employed thiolated peptides of the conformationally constrained, strongly helicogenic α -aminoisobutyric acid (Aib) residue to prepare self-assembled monolayers (SAMs) on gold surfaces. Electrochemistry and infrared reflection absorption spectroscopy support the formation of very well packed Aib-peptide SAMs. The immobilized peptides retain their helical structure, and the resulting SAMs are stabilized by a network of intermolecular H-bonds involving the NH groups adjacent to the Au surface. Binary SAMs containing a synthetically defined glycosylated mannose-functionalized Aib-peptide as the second component display similar features, thereby providing reproducible substrates suitable for the controlled display of bioactive carbohydrate ligands. The efficiency of such Aib-based SAMs as a biomolecular recognition platform was evidenced by examining the mannose-Concanavalin A interaction via surface plasmon resonance biosensing.

Keywords

Electrochemical desorption; glycopeptides; biosensors; self-assembled monolayers; Concanavalin A; surface plasmon resonance

INTRODUCTION

Carbohydrate-mediated biomolecular interactions play a variety of roles in biological systems, including cell-cell signaling, host-pathogen interactions and tumor progression.^{1,2} The display of carbohydrate ligands on cell surfaces directly affects the binding strength and specificity of carbohydrates to other biomolecules.³ To interrogate these interactions, a biosensing platform with stable and tunable bioactivity is desired.⁴ Biosensors are engineered to target an analyte present in a sample and transduce the binding event as a quantifiable output, usually via an optical or electrochemical signal. Here we will describe our findings on the preparation, properties and performance of a novel, robust and reproducible platform based on conformationally constrained peptides of α -aminoisobutyric

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Supporting Information

Experimental details about chemicals, syntheses, gold preparation and SAM formation, XPS, and IRRAS and SPR analyses. This material is available free of charge via the Internet at <http://pubs.acs.org>.

acid (Aib), and suitable for functionalizing surfaces with carbohydrates and studying biomolecular recognition.

Substrates suitable for biosensing can be obtained by using the strategy of self-assembled monolayers (SAMs) obtained via the adsorption of thiolated molecules onto gold substrates.⁵ The nature, packing, stability and, for electrochemical biosensors, the electron-transfer properties of functional SAMs are of paramount importance in determining the performance of the sensor itself. Since the length (and thus SAM thickness) and composition (suitable moieties exposed to solution) of the thiolated molecule can be controlled, SAMs facilitate rapid and tunable functionalization of sensor surfaces. Monolayer formation is driven by the chemical bonding between sulfur and gold atoms and by interactions between neighboring adsorbate molecules. In most cases, adsorbates interact primarily through intermolecular van der Waals forces, but additional stability can be achieved through intermolecular hydrogen bonds, e.g., between embedded amide groups. In this framework and because of their particular compatibility with biomolecules, peptides appear as ideal SAM building blocks.

Peptides possessing quaternary α -carbons, such as Aib oligopeptides, are strongly heliogenic and remarkably rigid.⁶ Due to steric hindrance at the α -carbon and restricted torsional freedom, these peptides form oligomers with a 3₁₀-helical secondary structure.⁷ Rigidity is provided by intramolecular C=O $\cdots$ H-N hydrogen bonds. To form a single helical coil, 3.24 amino-acid residues are required, and thus the 3₁₀-helix is more elongated and thinner (vertical pitch = 1.95 Å/residue) than the α -helix (vertical pitch = 1.55 Å/residue).⁶ It is worth noting that whereas the peptides of coded α -amino acids form stable helices only with long oligomers, the tendency of Aib homopeptides to give rise to structured peptides is already evident in very short oligomers.⁶ We previously used thiolated Aib-peptides to make monolayer-protected gold clusters (MPCs) and found that very stable 1-2 nm size MPCs can be prepared.⁸ While Aib-oligopeptides retained their 3₁₀-helical structure, MPC stability was granted by formation of a strong H-bond network connecting neighbor peptides in the capping monolayer. These MPCs are stable even after several years. Aib peptides also display outstanding properties as mediators for electron tunneling,⁹ a key feature for making electrochemical biosensors. Since MPCs are the 3D equivalent of the 2D SAMs formed on extended gold surfaces, we anticipated that such peptides could be ideal building blocks for making stable functional platforms for biomolecule recognition. The peptides examined in this work, thiolated on the N-terminus, are shown in Scheme 1.

Here we use electrochemistry and infrared reflection absorption spectroscopy (IRRAS) to show that thiolated Aib peptides form well-packed SAMs and are particularly valuable for making robust substrates suitable for functionalizing gold surfaces with bioactive carbohydrates. The ability of mixed Aib-SAMs to display bioavailable carbohydrate ligands was evidenced by studying the mannose-Concanavalin A (ConA) interaction via surface plasmon resonance (SPR).

EXPERIMENTAL

Full information on chemicals, peptide syntheses, gold surfaces preparation, electrochemical methodologies, X-Ray photoelectron spectroscopy (XPS), and IRRAS and SPR analyses is provided in the Supporting Information.

Electrochemistry

Peptide-SAM Au electrode measurements were conducted at 25 °C under an argon atmosphere using a CHI 660 C electrochemical workstation was used. A mercury oxide electrode (MOE) was employed as the reference electrode; its potential was -132 mV vs the

saturated calomel reference electrode. A Pt ring was used as the counter-electrode. The reductive desorption CV curves were recorded at a scan rate of 0.05 V s^{-1} . After SAM desorption, the electrochemical area of each working electrode was determined by running CV experiments at $0.1 - 1 \text{ V s}^{-1}$ in 0.5 M KCl containing 3 mM hexammineruthenium(III) chloride, whose diffusion coefficient is $6.84 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$. The roughness factor was calculated from the ratio between the electrochemical and the geometrical areas, and found to be 1.16 ± 0.08 .

Infrared Reflection Absorption Spectroscopy

The peptide SAMs were characterized with IRRAS and phase-modulation IRRAS (PM-IRRAS) measurements. IRRAS was carried out using a FT-IR Nicolet Nexus spectrometer at room temperature, equipped with a liquid nitrogen cooled Mercury-Cadmium-Tellurium (MCT) detector. The grazing-angle was set to 80° from the surface normal, which gives the maximum mean square electric field strength for the air/Au interface. The spectra were taken with a resolution of 2 cm^{-1} . Each spectrum was the average of 1000 scans, using an untreated gold plate as the reference, and carried out in a dry nitrogen atmosphere. PM-IRRAS measurements were carried out using a ThermoFisher Scientific Nicolet 6700 FT-IR spectrometer equipped with a Tabletop Optical Module for PM-IRRAS, an MCT-A detector (TRS 50 MHz, Nicolet, Madison, WI), a photoelastic modulator (PM-90, with II/ZS50 ZnSe 50 kHz optical head, Hinds Instruments, Hillsboro, OR), and a synchronous sampling demodulator (GWC Instruments, Madison, WI). Typically 1500 scans were performed with a resolution of 4 cm^{-1} .

Surface Plasmon Resonance

Protein-carbohydrate binding assays were performed on a SPRImagerII (GWC Technologies). The SPRImagerII was operated at room temperature using a standard flow cell and a peristaltic pump (BioRad-EconoPump) at $100 \mu\text{l min}^{-1}$. All surfaces were passivated with 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS, pH 7.4) for 30 min. ConA ($50\text{--}500 \text{ nM}$) was dissolved in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (10 mM HEPES, 150 mM NaCl, 1 mM Ca^{2+} and Mn^{2+} , pH 7.4). In a typical binding experiment, the peptide-SAM surface was equilibrated for ca. 10 min in HEPES buffer to stabilize the baseline signal, followed by passing ConA solution over the surface for ca. 8 min. HEPES buffer was subsequently flowed over the surface to facilitate dissociation for another 10 min. 8 M urea was used to strip bound protein and regenerate the SAM surfaces. All SPR binding curves were normalized by subtracting the background signal, represented by the sensor substrate passivated with BSA.

RESULTS AND DISCUSSION

To study the properties and packing of the SAMs, and to avoid specific interaction with aqueous media, we terminated the peptide with a *N-tert*-butyl amide. For this part of the study, we focused on peptides **1-3**, which form 310-helical structures with three to five intramolecular C=O $\cdots$ H-N hydrogen bonds. The bioactive carbohydrate functionalized peptide **4**, prepared as described below, had three intramolecular H-bonds. Formation of reproducible and well-packed Aib-based peptide SAMs requires careful cleaning and annealing of the gold substrate (Supporting Information). The peptide SAMs were prepared by incubating freshly annealed gold plates in 0.5 mM solutions of the appropriate peptide for 48 h.

The stability of the peptide SAMs was assessed by cyclic voltammetry (CV) in aqueous 0.1 M NaOH , under anaerobic conditions. When the electrode potential (E) is scanned negatively from -0.1 V vs MOE (mercury oxide reference electrode), a sharp reductive

desorption peak is detected (Figure 1). Desorption is essentially complete after one negative-going scan, as confirmed during further CV cycles. It is known that the potential and peak shape at which the reductive desorption occurs depends on the type and strength of interactions between the adsorbates and the properties of the metal surface.^{5,10} The fact that the peak potentials of **1-3** are the same within error (-0.845 ± 0.010 V) points to similar packing densities and electrochemical stability of these SAMs. The integral of the current density (*j*) corresponds to the overall charge associated with the desorption process. By making the simple assumption that one electron per molecule is consumed during reductive desorption,⁵ we calculated 3.0 ± 0.3 , 2.8 ± 0.5 , and $2.6 \pm 0.3 \times 10^{14}$ molecules cm^{-2} for **1**, **2**, and **3** respectively. Although significantly smaller than those of the thinner alkanethiols, ca. 5×10^{14} molecules cm^{-2} ,^{11,12} these values are high, and representative of tightly-packed SAMs. Based on a careful analysis of the factors affecting the desorption charge of SAMs, Lipkowski and co-workers showed that, in fact, the above procedures overestimate by ca. 15% the actual surface coverage.¹³ Even factoring in this important correction, the above packing values are significantly larger than expected based on the physical requirements of the 310-helix.¹⁴ We attribute this outcome to the presence of a strong dipole moment associated with the 310-helical structure,¹⁵ which is expected to affect the calculation¹³ of the actual desorption charge. For the sake of the current investigation, however, the above results point to very well packed SAMs, as also found for analogous Aib-peptide SAMs on mercury.¹⁴ Equally important is the fact that the peptide length does not display any evident effect on coverage. Based on its desorption behavior, glycopeptide **4** also forms a virtually identical SAM, the apparent coverage being $2.9 \pm 0.3 \times 10^{14}$ molecules cm^{-2} . A slightly more negative peak for **4**, -0.872 ± 0.002 V, is in keeping with the formation of further H-bonds between the mannose moieties.

IRRAS and PM-IRRAS were used to provide information on the helical structure of the peptide in the SAM. Figure 2a shows the amide A region of peptides **1-4** in the SAMs and that of the corresponding trityl-protected peptides in solution (inset). In a poor H-bond acceptor solvent such as CH_2Cl_2 , the band at 3420 cm^{-1} pertains to the stretch of free NH bonds which, based on the 310-helix features, correspond to the first two NH groups on the N-terminus.^{7,16} This peak, however, is absent in the IRRAS spectra of Aib-peptide SAMs: similarly to the same peptide SAMs on gold nanoclusters,⁸ this indicates that all NH groups are involved in inter- and/or intramolecular hydrogen bonds. The band at lower energy is due to such H-bonded NH groups (for the peptides in solution, only intramolecular H-bonds): as the peptide is made longer, the intensity of this band increases and undergoes a red-shift toward lower frequencies, regardless of whether the peptide is in solution or in a SAM, a fact attributed to formation of progressively more stable helices.^{7,8,16} Concerning glycopeptide **4**, the observed IRRAS band results from the overlap of the NH band (similar to that of peptide **1**) with that of the O-H stretching vibrations of the hydroxyl groups of mannose; for example, in a SAM composed by mannose-functionalized alkanethiols, the latter has been reported as a broad peak at 3334 cm^{-1} .¹⁷ Our observation of a band at ca. 3312 cm^{-1} , which is red-shifted also relative to that of peptide **1** (ca. 3322 cm^{-1}), points to formation of a particularly stiff monolayer. The conclusion reached for peptide SAMs implies that the first two NH groups of the 310-helix are hydrogen bonded to neighboring peptides' C=O groups that are already involved in intramolecular H-bonds. These three-center H-bonds allow formation of a strong interchain bonding network and, since they are embedded inside the monolayer, they are largely unaffected by an increase of the peptide length.

As the peptide length increases, the amide I band of peptide SAMs, $1675\text{-}1676 \text{ cm}^{-1}$ (Figure 2b), and trityl-protected peptides, $1664\text{-}1674 \text{ cm}^{-1}$ (inset), which is mostly due to the C=O stretch, undergoes a similar absorbance increase as observed for amide A of **1-3**. Figure 2b shows that the peptides behave very similarly in solution and in a monolayer. Based on very

recent findings, the upshift of the amide 1 wavenumber observed as one goes from solution to SAM can be attributed to vibrational coupling between amide groups of nearby helices.¹⁸ Since the irradiated area and the surface roughness factor were kept constant, the fact that the intensity of the main PM-IRRAS bands are an increasing function of the peptide length suggests similar peptide coverage, also in agreement with the reductive desorption studies. Based on the frequency values and peptide length dependence,^{7,16} both IR regions thus point to SAMs formed by peptides with helical structures displaying pronounced 310-helical character, as observed in solution phase. As for amide A, the amide 1 band of glycopeptide **4**, centered at 1674 cm⁻¹, points to a slightly stiffer system. Amide 1 and amide 2 (the broad band around 1530-1540 cm⁻¹ in Figure 2b) are particularly sensitive to the tilt of the peptide axis with respect to the surface normal, in the sense that the more vertical the peptide, the larger the intensity of the amide 1 band with respect to that of amide 2. This effect makes the two intensities quite different from those observed in solution. Keeping in mind the approximations required in such calculations, analysis of the amide 1 and amide 2 absorbance ratio using an orientation distribution function model¹⁹ (Supporting Information) shows that as the peptides get longer the molecules assume a more upright position: for peptides **1-4**, the tilt angle is calculated to be approximately ($\pm 5^\circ$) 47, 39, 28, and 30°, respectively.

The electrochemical and IR results thus point to very well packed Aib-peptide monolayers stabilized by intermolecular H-bonds involving the NH groups closer to the Au surface, and with peptides still preserving their helical structure while adopting an upright orientation relative to the gold surface. Because of these features and particularly important within the aims of the present study, increasing the length of the peptide by one or two amide units does not significantly change the monolayer's properties and stability. For these reasons, we have selected to examine a single length of representative thiolated glycopeptides, compound **4**, which contain three intramolecular H-bonds as **1**.

Leveraging the well-packed Aib peptide monolayers, we investigated the effects of the glycopeptide/peptide mixed SAMs on carbohydrate-protein interactions utilizing the SPR approach. A series of mixed SAMs consisting of glycopeptide **4** and unfunctionalized peptide **1**, used as the diluent, were formed on 45 nm gold substrates by varying the relative concentration of the two peptides in 1:1 water-ethanol solutions. XPS was employed to characterize the mixed peptide SAMs, observing the expected chemical species. Whereas the S/N ratio did not change (same amount of S and N atoms in the two molecules), the O/N ratios increased with the concentration of glycopeptide **4** (Figure S8). These results indicate that the surface density of carbohydrate ligands is readily tuned by varying the solution ratio of Aib glycopeptides to non-glycosylated Aib peptides. In the following, we will refer to the nominal concentration values.

To examine the bioactivity of these mixed SAMs, SPR analysis was performed. Using a silicone mask, we prepared a single SPR chip that contained multiple discrete SAMs consisting of different concentrations of glycopeptides (10-100%, diluted with **1**) and subsequently detected their bindings to the mannose-binding protein ConA. As illustrated in Figure 3, which pertains to a 500 nM ConA solution, whereas the SAMs containing 10-100% mannose-functionalized glycopeptides demonstrate specific binding to ConA, the non-glycosylated peptide does not. In control experiments (see Supporting Information) we verified that the 50 and 100% mannose-functionalized peptides do not show any reactivity toward the galactose-binding protein Ricin. We did, however, find that Ricin bound nicely to SAMs containing a galactose-containing peptide (Figure S1). We also verified that changing the diluent peptide **1**, which is NHtBu protected on the carbon terminus, with its free acid analog, did not significantly alter the overall SPR behavior (Figure S2).

The mannose-ConA binding interaction was analyzed by studying the kinetics of the ConA adsorption-desorption SPR patterns (cf Figure 3 and Figure S3) recorded as a function of both the ConA concentration and the nominal percentage of the mannose-functionalized peptide in the SAM. The association rate constant, k_a , and dissociation rate constant, k_d , were obtained as described in the Supporting Information, and the association equilibrium constant, K_a , was obtained from the ratio k_a/k_d . K_a was found to vary from ca. $2 \times 10^6 \text{ M}^{-1}$ at low glycopeptide **4** coverage to ca. $4 \times 10^6 \text{ M}^{-1}$ at high coverage (Figure S4). These K_a values are thus in agreement with values reported for other glycosylated SAM systems, ranging from 10^6 to 10^7 M^{-1} .^{17,20} (for other references, see Supporting Information). The observed trend is qualitatively in keeping with a possible transition between 1:1 to 1:2 bindings, as already discussed.²⁰

OUTLOOK

Comparison of the electrochemical, IRRAS, XPS and SPR results shows that Aib-peptides and corresponding glycopeptides form well-packed SAMs with tunable bioactivity. These glycopeptide-based systems thus provide reliable substrates that can be used to functionalize biosensors or other surfaces for studying biomolecule recognition. Our previous studies on mixed SAMs consisting of thiolated ethylene-glycol-modified glycans and an ethylene-glycol thiol exhibited similar binding behaviors but also highlighted significant time-dependent phase-separation phenomena.^{21,22} This is indeed a quite general problem for binary-SAMs.^{5,23,24} In fact, the tendency of mixed SAMs to phase-separate at the nanoscale represents a potential challenge toward molecular engineering of controlled glycan densities in mixed glyco-SAM systems. This is conceivable based on the known self-associative property of carbohydrates,^{25,26} a fact that must be taken into account in glycan sensor and array design. The approach described here, which leverages the self-associative properties and robustness of Aib-oligopeptides in SAMs, may provide a turning point in designing stable and bioactive sensor substrates, also as an alternative to other methods, such as immobilization by biotin-tagged oligosaccharides.²⁷⁻³⁰ The outcome of the SPR study together with the IRRAS evidence of formation of an intermolecular H-bond network holding together neighbor peptides still retaining their helical structure, point to these mixed SAMs as not only very well packed but also as stable systems toward phase-separation. Further investigation on this aspect as well as other glycan-based sensor systems are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Voltammetric desorption peaks of the SAMs obtained from peptides **1-4** on Au. The curves were obtained at 0.05 V s^{-1} in 0.1 M NaOH aqueous solution. $T = 25 \text{ }^{\circ}\text{C}$.

Figure 2. IRRAS spectra of the amide A (graph a) and PM-IRRAS spectra of amide 1 and 2 bands (graph b) for peptide SAMs of **1** (blue curve), **2** (red curve), **3** (black curve), and **4** (green curve) on Au. The two insets show the corresponding FT-IR absorption spectra obtained with the trityl precursors (1 mM) in CH₂Cl₂.

Figure 3.

Time dependence of the SPR response of 500 nM ConA, in HEPES buffer, to the SAMs containing thiolated mannose-peptides **4** and peptides **1**. The curves correspond to the following percentages of **4** in the incubating solutions (bottom to top): 0, 10, 25, 37.5, 50, 62.5, 75, 90, and 100%. The decreasing curves correspond to washing the surfaces with HEPES buffer.

Scheme 1.

Peptides used to prepare the SAMs. Dashed lines illustrate H-bonds based on the 310-helical structure.