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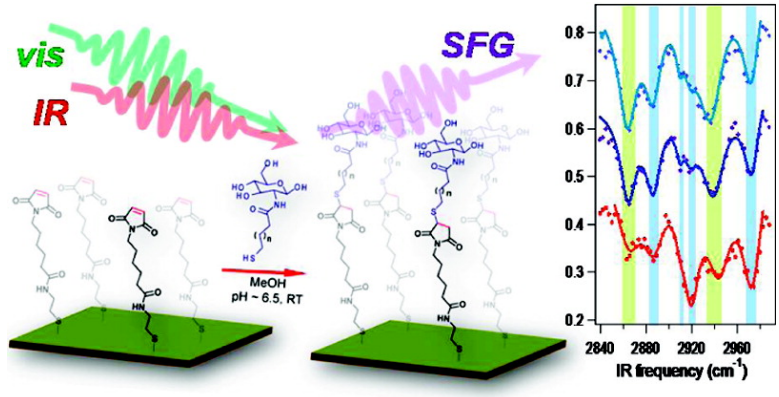

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Carbohydrate Surface Attachment Characterized by Sum Frequency Generation Spectroscopy

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Covalent surface attachment of carbohydrate moieties using maleimide-sulfhydril reaction was characterized by surface-selective vibrational sum-frequency generation (VSFG) spectroscopy. The comparative VSFG spectra of the precursor maleimide-terminated SAM and the product glucose adlayer reveal the high efficiency of the surface coupling reaction (>90%) and the details of the molecular organization of the formed carbohydrate adlayer. The glucose groups are orientationally well ordered, as judged by their sharp C−H stretch bands. The chemical structure of the linker can significantly affect the orientation of the carbohydrate moiety at the surface. Two alkanethiol linkers of different chain lengths (11 and 16 carbons) yield similar orientations of the glucose in the adlayer whereas the cysteine-containing linker produces markedly different relative peak intensities of the glucose C−H stretch bands in the VSFG spectra, suggesting a significantly different orientation with respect to the surface plane.

Carbohydrates immobilized on solid surfaces have utility in both fundamental glycobiology research and in multiple biomedical and biotechnology applications such as disease diagnostics, bioactive/biocompatible surface coatings, biosensors, and the detection of bioterrorism threats.[1−6] Because of their ubiquity in living cells and their diversity of interactions with other biomolecules, carbohydrates offer a new realm of opportunities to address cellular events, molecular recognition, protein binding, high-throughput screening techniques for carbohydrate−biomolecule interactions, and the development of therapeutic agents.[7−11] Surface immobilization is also a useful tool in combinatorial chemistry for the spatial encoding[12] of libraries of novel oligosaccharides.

Covalent attachment is the preferred method for surface functionalization because it produces controllable, stable, and reusable adlayers.[13−16] Whereas many strategies for covalent attachment to create carbohydrate chips and microarrays are being adapted from the well-developed bioorganic synthetic methodologies,[4,17,18] in situ characterization of the coupling reaction efficiency and molecular structure/order of the resulting monolayers remains a challenge. Yet the molecular organization (e.g., orientation of sugar moieties on surfaces) is of critical importance to the intended function (e.g., protein binding, chemical reactivity,[19] or catalytic activity).

In this report, we demonstrate the utility of surface-selective nonlinear optical spectroscopy for monitoring the surface covalent attachment reactions and dissecting the molecular structure of the created adlayers. Vibrational sum frequency generation (VSFG), which is surface-selective nonlinear optical spectroscopy,[20−22] has emerged as a powerful tool that yields a wealth of information on the molecular orientation and conformation at interfaces,[15,23−26] including biomolecules such as phospholipids,[27−30] proteins,[31,32] and DNA.[33,34] Here we apply VSFG to monitor the covalent surface attachment of monosaccarides by the maleimide-sulfhydril coupling reaction and characterize the molecular structure of the created adlayers.

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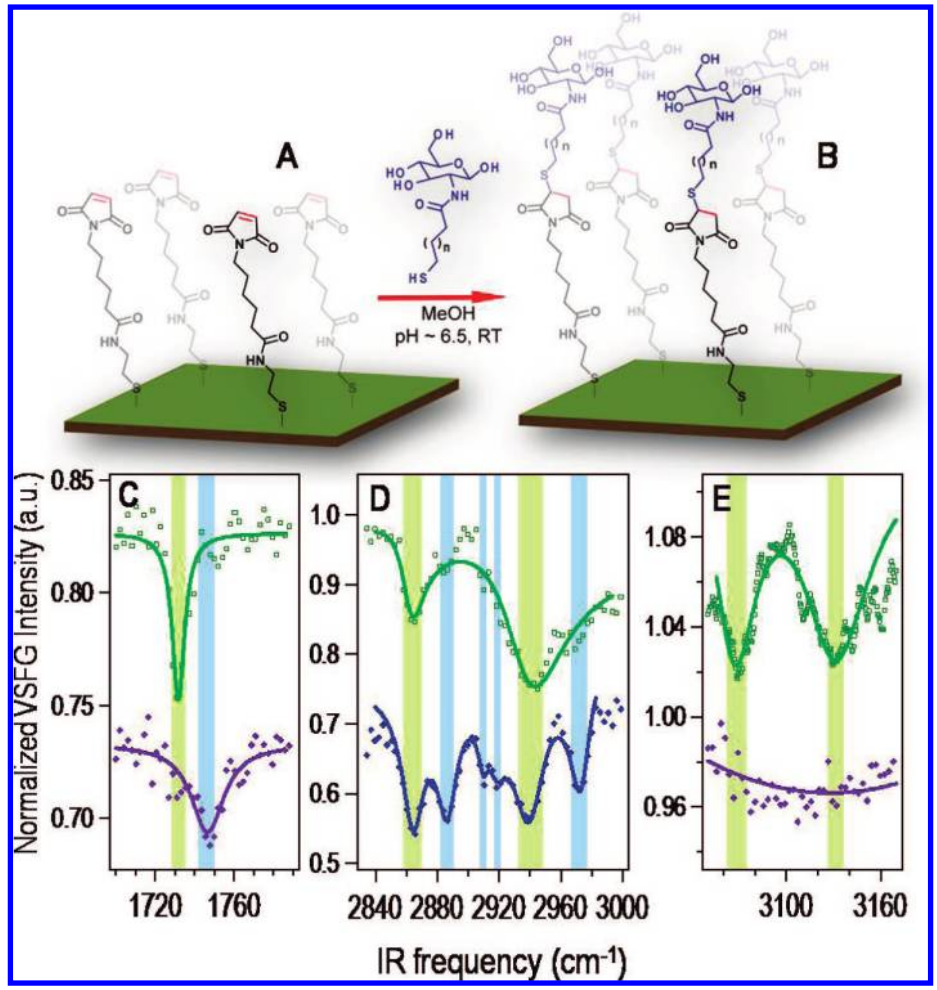

Figure 1. (A) Precursor maleimide-terminated SAM. (B) Glucose-terminated SAM produced by covalent surface attachment. (C−E) Vibrational SFG spectra of the precursor SAMs (green, top traces) and product carbohydrate adlayers (blue, bottom traces) in three frequency regions: (C) C═O stretch of the maleimide group, (D) saturated C−H stretch, and (E) unsaturated C−H stretch of the maleimide group. Green shades highlight the characteristic bands of the precursor maleimide SAM; blue shades highlight the bands of the product carbohydrate SAM. Spectra are vertically offset for clarity.

Nucleophilic substitution reactions are often employed for surface chemical modification. In particular, maleimide-terminated self-assembled monolayers (SAMs) were investigated recently[14,16,35] because maleimide groups are readily available, stable, can be easily derivatized, and yet can selectively react with sulfhydril or amine functionalities with high yield under mild conditions.[17] This has been exploited in creating a variety of biochips including DNA, peptides, and carbohydrates.[1,16,35]

As in other studies,[16,35] we utilized alkanethiol self-assembly on a gold surface[36,37] to produce the precursor maleimide-terminated scaffold (Figure 1A). We note, however, that the VSFG technique does not require a metal surface and can be applied to other substrates (e.g., glass slides using silane groups for attachment).[15,38]

The maleimide-terminated precursor SAMs (Figure 1A) were prepared on gold slides by incubation in a 2 mM methanolic (99% anhydrous) solution of *N*,*N*′-bis(maleimidylhexanoyl)cystamine (>99.9%) for 48 h at room temperature and characterized by VSFG spectroscopy (Figure 1C−E). Subsequently, covalent surface functionalization was performed with a monosaccharide derivatized with a sulfhydril group on alkane tethers (Figure 1B) or with a cysteine linker as a glycopeptide-like mimetic motif to be used in future binding studies. We studied three different adlayers (Scheme 1) to investigate the effect of the linker length and chemical composition on the molecular structure of the adlayer. We have also performed control experiments in which a short-chain (C_6) alkane thiol was covalently attached to the same precursor maleimide-terminated SAM.

The synthetic procedure is outlined in Scheme 1, and the details are presented in the Supporting Information. Briefly, 2-amino-2-deoxy-D-glucose hydrochloride (**1**) was coupled to 11-tritylthioundecanoic acid and 6-tritylthiohexanoic acid employing standard HOBt/HBTU reagents. Removal of the trityl groups using TFA revealed compounds **2** and **3** in excellent yield (92%). Coupling of **1** with 4-(*N*-Boc-amino)butanoic acid followed by Boc removal yielded **4**, which was then coupled to *S*-trityl-*N*-Boc-cysteine to yield **5**. Under acid conditions, both protecting groups were simultaneously removed to reveal **6**. Vibrational spectra in three fingerprint regions—the carbonyl stretch of the maleimide group, the saturated C−H stretch region, and the unsaturated C−H stretch of the maleimide group (Figure 1C−E) were recorded before and after surface functionalization to characterize the molecular organization of the precursor maleimide SAMs, the extent of the covalent surface attachment, and

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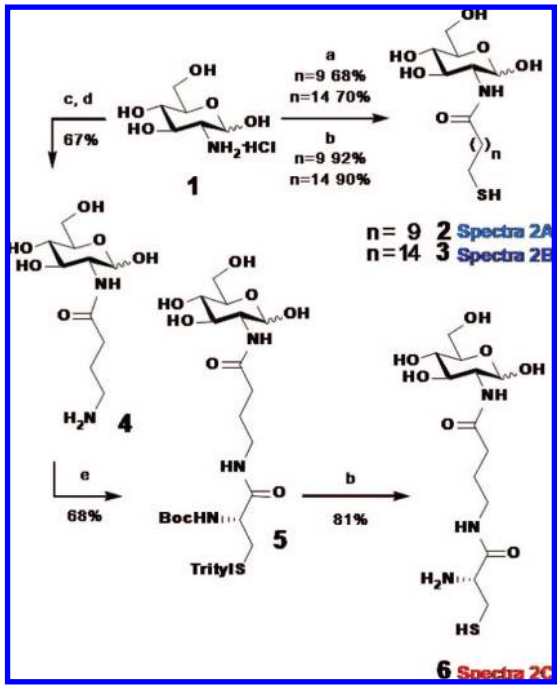
Scheme 1. Synthesis of Glucose Derivatized with Alkanethiol Linkers C_{11} (2) and C_{16} (3) and a Cysteine Linker (6)[a]



[a] Conditions: (a) $HO_2C(CH_2)_{10}S$-trityl/ $HO_2C(CH_2)_{15}S$-trityl, HBTU, HOBT DIPEA, DMF, DMSO. (b) TFA, TIPS, 0°C. (c) $HO_2C(CH_2)_3NHBOC$, HBTU, HOBt DIPEA, DMF, DMSO. (d) TFA, anisole, 0°C. (e) *S*-trityl-*N*-Boc-L-Cys, HBTU, HOBT DIPEA, DMF, DMSO.

the structure of the formed carbohydrate adlayer. All spectra were recorded using the PPP (SFG−vis−IR) polarization combination. Because of the nonresonant background from the gold substrate, the vibrational resonances appear as negative peaks in the spectra.[39] We use broadband VSFG spectroscopy[39,40] to acquire vibrational spectra by tuning the broadband (~250−300 cm^{-1}) IR pulse to the spectral region of interest. Our setup,[25,41] the data acquisition procedure, and the spectral fitting procedure are described in detail in the Supporting Information.

The VSFG spectra of the precursor maleimide-terminated SAMs display sharp vibrational bands: the C═O stretch of the α-β unsaturated maleimide group at 1728 cm^{-1} (Figure 1C, top trace), the unsaturated C−H stretch (symmetric and asymmetric) of the maleimide at 3066 and 3130 cm^{-1} (Figure 1E, top trace), and the symmetric and asymmetric CH_2 stretches of the alkane chain at 2862 and 2937 cm^{-1} (Figure 1D, top trace). Qualitatively, this indicates a high degree of order and orientational alignment of the maleimide SAMs, characteristic of other thiol SAMs on gold substrates.[24,42,43] The SFG signal vanishes in the limit of orientationally disordered molecules on a surface. Pronounced changes in the SFG spectra upon chemical modification clearly indicate the extent of the covalent surface attachment reaction. The C═O stretch of the unsaturated maleimide ring blue shifts to 1746 cm^{-1} for the saturated ring[16,44] (Figure 1C). Note that the amide I carbonyl stretches usually appear below 1700 cm^{-1}

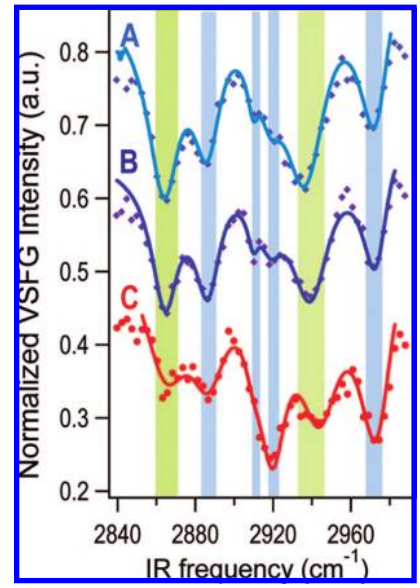


Figure 2. VSFG spectra in the saturated C−H stretch region of (A) C_{11}-glucose **2**, (B) C_{16}-glucose **3**, and (C) cysteine-glucose **6** adlayers. Green shades highlight the bands of the precursor maleimide SAM (Figure 1D); blue shades highligh the glucose C−H stretch bands of the adlayer. Spectra are vertically offset for clarity.

(on the basis of FTIR data in literature),[16,45] and we thus assume that the amide stretches of the linkers for both the maleimide precursor SAM and the glucose tether do not contribute to the spectra in Figure 1C. The unsaturated C−H stretches of the maleimide group disappear completely after the reaction (Figure 1E); the CH stretch of the saturated penta-ring after the coupling is expected at around 2900 cm^{-1}. Peak amplitudes from fitting the SFG spectra using multi-Lorentzian functions (solid lines) allow us to estimate the yield of the surface attachment reaction as >90%. Fitting parameters are presented in the Supporting Information.

In the saturated C−H stretch region, new transitions at 2887, 2910, 2919, and 2974 cm^{-1} (Figure 1D) appear after the maleimide-sufhydril reaction, which can be assigned to the C−H stretches of the glucose ring.[46] These are distinct from the CH_2 modes of the linker and the precursor SAM, which occur at 2852 and 2925 cm^{-1} as in their bulk-phase FTIR spectra (presented in Supporting Information). The glucose C−H bands are well pronounced, with an amplitude and width similar to those of the precursor SAM bands, suggesting that the glucose adlayer formed by surface covalent attachment is well-ordered.

The disappearance of the characteristic maleimide unsaturated C−H bands and frequency shift of the maleimide C═O bands after the surface attachment reaction was observed for all tested adlayers—the glucose with two alkanethiol linkers **2** and **3** of different lengths, C_{11} and C_{16}, the cysteine-based linker **6** (Scheme 1)—as well as in the control experiment with a short alkane thiol (C_6) attachment.

A quantitative analysis of the glucose group orientation based on the C−H stretch VSFG spectra would require a knowledge of the $\beta^{(2)}$ hyperpolarizability tensor elements for the glucose C−H stretches, which are not currently available. Nevertheless, a qualitative comparison of the VSFG spectra of the glucose C−H stretches for alkanethiol linkers of two different lengths (**2** and **3**, C_{11} and C_{16}) shows very similar relative amplitudes of

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the four observed glucose C−H stretch modes (Figure 2, curves A and B). This indicates that the projections of these four vibrational modes onto the surface coordinate system are similar (i.e., the orientation of the glucose group is the same for the two alkanethiol linkers), with no pronounced even−odd effect[42] on the orientation.

To explore the surface attachment of glycopeptide-like moieties, we compared alkanethiol linkers **2** and **3** with a shorter linker **6** containing a cysteine moiety (Figure 2, curve C). The VSFG spectra of cysteine-linker-functionalized SAMs show quite different glucose bands (Figure 2). Whereas the same C−H stretch bands of the precursor maleimide SAM are observed in all three spectra (highlighted by light-green shading), notable differences are apparent in the glucose SFG bands of the product adlayer. The glucose C−H stretch transitions, highlighted by light-blue shading in Figure 2, are observed at the same frequencies, but their relative amplitudes are very different in the case of the cycteine linker. The VSFG amplitude of a given vibrational mode is determined by (1) its second-order hyperpolarizability tensor $\beta^{(2)}$ and (2) its orientation in the surface-coordinate frame. Because the different C−H stretch modes of glucose likely have different $\beta^{(2)}$ tensors, their relative amplitudes are determined by the orientation of the glucose in the surface frame. We therefore conclude that the orientation of the glucose group attached via the cysteine-containing linker is significantly different from alkanethiol linkers.

In summary, we have characterized the covalent surface attachment of carbohydrate moieties using the maleimide-sulfhydril linkage by monitoring the characteristic vibrational transitions of the reactant maleimide SAM and the resulting product, the glucose adlayer. Surface-selective VSFG spectroscopy reveals the high efficiency of the surface attachment reaction (>90%) and the high degree of molecular organization of both the precursor maleimide SAM and the formed carbohydrate adlayers. The orientation of the glucose group with respect to the surface plane is the same for two alkanethiol linkers of different lengths but distinctly different for a cysteine linker. The chemical structure of the linker can therefore significantly affect the orientation of the sugar moiety at the surface. This conclusion may have important mechanistic consequences in many applications of carbohydrate-functionalized surfaces such as protein and glycoprotein binding, carbohydrate−biomolecule interactions, and molecular recognition. The surface-selective vibrational SFG spectroscopy approach described here is capable of yielding details of the molecular organization that will be important in future studies of other monosaccharides and disaccharides at surfaces, as well as for antibody binding studies.

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Supporting Information Available: Synthesis procedures and analytical data for all compounds and intermediates; $^{1}H/^{13}C$ NMR; details of the VSFG setup and analysis of the VSFG spectra, fitting parameters, and peak assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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