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Characterization of Surfaces Presenting Covalently Immobilized Oligopeptides using Near-Edge X-Ray Absorption Fine Structure Spectroscopy

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Abstract

This study addresses the need for methods that validate the surface chemistry leading to the immobilization of biomolecules and provide information about the resulting structural configurations. We report on the use of near edge x-ray absorption fine structure spectroscopy (NEXAFS) to characterize a widely employed immobilization chemistry that leads to the covalent attachment of a biologically relevant oligopeptide to a surface. The oligopeptide used in this study is a kinase substrate of the epidermal growth factor receptor (EGFR), a protein that is a common target for cancer therapeutics. By observing changes in the π^* and σ^* orbitals of specific nitrogen and carbon atoms (amide, imide, carbonyl) we are able to follow the sequential reactions leading to immobilization of the oligopeptide. We also show that it is possible to use NEXAFS to extend this characterization method to sub-monolayer densities that are relevant to biological assays. Such an element-specific chemical characterization of small peptides on surfaces fills an unmet need and establishes NEXAFS as useful technique for characterizing the immobilization of small biomolecules on surfaces.

Introduction

Control of the structure and associated functions of biomolecules at interfaces underlies the successful design of solid-state biosensors, DNA microarrays, and protein chips¹. To this end, the development of methods that permit characterization of these interfaces and their chemical reactions represents an important challenge. Currently, relatively few techniques exist for the characterization of biomolecules immobilized in monolayer and sub-monolayer coverages at interfaces. Methods that are widely used to characterize the structure of biomolecules in bulk solution (such as NMR and CD) are not readily applied to surfaces, because the number of molecules at a surface is too low to generate an adequate signal. Existing methods that do permit characterization of surface-immobilized biomolecules include vibrational and x-ray spectroscopy². Vibrational methods, such as infrared (IR) spectroscopy and infrared-visible sum frequency spectroscopy (SFS), probe molecular vibrational states that characterize particular bonds. Polarized Fourier Transform IR methods can be used to characterize the structure of peptides^{3, 4}. However, they are not surface selective, and deconvolution of IR spectra can be difficult for small peptides that lack secondary structure. Factors such as solvent conditions or changes in hydrogen bonding environment can also complicate interpretation of

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the spectra^{5, 6}. In contrast, SFS is surface-selective⁷, but the quantitative interpretation of the data is more difficult⁸.

In contrast to IR-based vibrational spectroscopy, x-ray spectroscopic techniques characterize the electronic structure of molecules at surfaces^{1, 9}. X-ray photoelectron spectroscopy (XPS) and near-edge x-ray fine structure spectroscopy (NEXAFS) both use incident x-rays to excite electrons from a specific core level, which makes them chemically selective. NEXAFS goes one step further and also identifies unoccupied π^* and σ^* molecular orbitals which can be assigned to specific bonds. NEXAFS becomes polarization-dependent for oriented, low-lying orbitals, while XPS excites into isotropic, high-lying final states. Thus, NEXAFS can probe both surface composition and the orientation of surface immobilized molecules¹⁰. In this paper, we report the use of NEXAFS to characterize reactions involved in the covalent immobilization of short sequences of peptides (oligopeptides) at surfaces, and provide insights into their orientation.

This study builds upon prior investigations that have used NEXAFS to characterize the composition and structure of biomolecules, such as amino acids, nucleic acids, and peptides^{2, 11–20}. For example, NEXAFS spectra of bulk samples of single amino acids and nucleic acids have been reported by Zubavichus et al. as parts of investigations that sought to enable determination of the composition of biomolecules containing multiple amino acids or nucleic acids^{13, 19, 21}. In addition, studies aimed at characterizing the conformations of amino acids have been performed with NEXAFS and x-ray photoemission spectroscopy^{13, 17, 19}. In particular, Polzonetti et al. studied peptides with repeating EAK residues (where E, A and K are the residues of glutamic acid, alanine and lysine, respectively) bound to TiO₂ via deprotonated carboxyl group of the constituent amino acids and characterized the orientation of the peptides relative to the surface via NEXAFS^{14, 22}. In a second study, Iucci et. al. showed that by scrambling the EAK sequence, NEXAFS spectra of peptides adsorbed to the surface no longer exhibited a preferred orientation^{14, 15}. Finally, we comment that we have reported previously the use of NEXAFS to characterize the conformation of a large protein (RNase A, molecular weight 13.7 kDa) immobilized at saturation coverage on surfaces by observing the polarization dependence of transitions associated with minority species such as S and N within the amino acid backbone of the protein¹⁸. Whereas these past studies demonstrate that NEXAFS can be used to characterize the orientations of nucleic acids²³, oligopeptides, and proteins on surfaces, herein we report that NEXAFS forms the basis of a useful tool to characterize widely employed reactions that lead to the covalent immobilization of biomolecules at surfaces as well as the resulting orientational states of the biomolecules. Because the orientational states of biomolecules of surfaces are strongly dependent on the immobilization chemistry, the capability to characterize both immobilization chemistry and orientational states of biomolecules should be a broadly useful one for studies of biomolecular interfaces.

The procedure used to immobilize the oligopeptide in our study involves two reactions that are widely employed for immobilization of biomolecules on surfaces: the first reaction is between a primary amine and an N-hydroxysuccinimide (NHS) ester; the second reaction is between a maleimide group and a free thiol. The oligopeptide is immobilized on a self-assembled monolayer that comprises two-components; one component presents tetraethylene glycol (EG₄), and is included in the design of the surface to resist non-specific adsorption of the oligopeptides. The second component of the SAM is an amine-terminated tetraethylene glycol (EG₄N). This group is incorporated into the design of the surface as a reactive moiety. By controlling the composition of the mixed SAM, the density of oligopeptides immobilized on the surface can be precisely controlled (as verified in our study)^{24, 25}. We note also that the immobilization of the peptide via the terminal cysteine group (reaction of the maleimide with the thiol) is a broadly applicable approach that can be applied to other peptide systems. In

addition, because our study employs an oligopeptide (14 amino acids), we demonstrate that it is possible to observe the orientations of peptides at the N1s and C1s edges of the NEXAFS spectrum rather than rely on the use of rare elements as was demonstrated for characterization of larger proteins such as RNase A18.

The peptide used in this study has high biological relevancy; it is a peptide substrate of the epidermal growth factor receptor (EGFR), a protein that is the target of a range of small molecule tyrosine kinase inhibitors^{26, 27}. While a number of past studies have employed surface immobilized kinase substrates, they have not characterized in detail the reactions that lead to the immobilization of the oligopeptides nor have they characterized the orientations of the peptides on the surface^{28, 29}. The oligopeptides used in our study have not been designed to adopt any particular secondary structure (unlike homopeptides and proteins that adopt helical structures). We note that the study of the structure of oligopeptides that lack secondary structure poses a unique challenge. Below we describe that it is possible to use NEXAFS to characterize the step by step immobilization chemistry of the oligopeptides, and we also find evidence that the peptides adopt a preferred orientation when immobilized on the surface. We characterized surfaces using a range of densities- from saturation coverage through densities that are relevant for bioassays based on the EGFR.

Materials and Methods

Materials

All materials were used as received unless otherwise noted. Tetra(ethylene glycol)-terminated alkanethiol (EG₄) and the corresponding amine-terminated alkanethiol (EG₄N) as a hydrochloride salt were obtained from Prochimia (Poland). The sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SSMCC) linker was obtained from Pierce Biotechnology (Rockford, IL). Silicon wafers were purchased from Silicon Sense (Nashua, NH). Ethanol was obtained from Sigma Aldrich (Milwaukee, WI) and purged with argon gas before use. Phosphate Buffered Saline (PBS) was made by diluting PBS packets purchased as a powder from Pierce Biotechnologies (Rockford, IL) and diluted in MilliQ water.

Oligopeptides were purchased in unphosphorylated and phosphorylated forms (Cys-Thr-Ala-Glu-Asn-Ala-Glu-[p]Tyr- Leu-Arg-Val-Ala-Pro-Gln) from New England Peptide (Gardner, MA).

MS analysis was performed by New England Peptide (and reported to be within 0.1% of the exact molecular weight. The purity of the peptides was found to be >95% as determined by HPLC analysis.

Preparation of Gold Films

Reflective gold films (10nm Ti and 200nm Au) were prepared by physical vapor deposition onto silicon wafers, as described in a previous study³⁰. All gold films were used within one week of preparation.

Preparation of Self-Assembled Monolayers and Oligopeptide-Modified Surfaces

Solutions containing mixtures of EG₄ and EG₄N (1mM total thiol concentration) were prepared in ethanol. Gold films were immersed in the EG₄/ EG₄N solutions for 18 hours, rinsed using copious quantities of water and ethanol, and dried under a stream of nitrogen gas. 2mM solutions of the heterobifunctional crosslinker SSMCC (in 0.1M PBS, pH 7.4) were deposited as droplets onto the mixed monolayers and incubated for 1.5 hours at room temperature. These surfaces were rinsed in ethanol and water and dried under nitrogen gas. Solutions of the cysteine-terminated EGFR peptide substrate (Y1173, 500μM) in water were then applied as

droplets. We used cysteine-terminated peptides as they site-specifically react with surface-immobilized maleimide groups introduced by using SSMCC. During reaction with the oligopeptides for 3 hrs, the substrates were stored in a chamber saturated with water. The choice of reaction times and buffer conditions were guided by previously published results^{24, 25, 31} and materials handling guidelines provided by Pierce³¹. We note that two thiols that differ substantially in structure can segregate within a mixed SAM formed on a gold film.^{32, 33} We comment, however, that the structure of the two thiols used to prepare the mixed SAMs in our study are similar, and thus it is unlikely that they substantially segregate on the surface. Ellipsometry, XPS, and AFM were used to characterize the oligopeptide decorated surfaces (see Supporting Information Figure S4).

Preparation of Bulk Samples

Bulk samples of EG₄N, SSMCC, and peptide were prepared by applying a droplet of solution (same solutions as described above for immobilization of the peptides) to a gold film and allowing the solvent to evaporate.

NEXAFS Measurements

The N 1s NEXAFS spectra were acquired on Beamline 8.0 at the Advanced Light Source (ALS) of the Lawrence Berkeley National Laboratory. Experiments were performed by measuring the electron yield (TEY), i.e. the total sample current, and the fluorescence yield (FY), both in normal incidence. The measurements of the FY were performed by a micro-channel plate (MCP) detector with an Al filter, which improved the signal-to-background ratio by a factor of 5–10 compared to TEY (see Fig. 4). The C 1s TEY spectra were taken at the VLS-PGM beamline of the Synchrotron Radiation Center (SRC) of the University of Wisconsin-Madison. While the electrons in TEY measurements come from within 5–10nm of the surface, the photons detected in FY have a longer attenuation length of 100nm–1 μ m, depending on the absorption length at the energy of the emitted photons (See Figure S1 for a schematic of the absorption events.) In our situation the probing depth is limited by the thickness of the organic film (about 5nm), i.e., the effective probing depth of TEY and FY are similar. Radiation damage effects were minimized by using very narrow monochromator slits. At the ALS, the beam was defocused to 5 $\times$ 5 mm². A series of subsequent spectra was taken on the same spot to observe radiation-induced changes, such as the appearance of C=C and C=N π^* orbitals from dehydrogenation. The homogeneity of the samples was confirmed by taking spectra at 2–3 separate spots and using multiple samples from the same batch. The raw data were first normalized to the photocurrent from a clean upstream Au mesh, in order to correct for temporal and spectral variations of the incident photon flux. The background signal measured at the pre-edge was fitted with a linear function and subtracted from the spectra. In a second normalization step we divided the spectra by the pre-edge background. This type of normalization quantifies the amount of molecules bound to the surface, assuming that the background is dominated by the Au substrate. In the case of physisorbed droplets (Figure 2A), the films are so thick that the electrons from the Au substrate cannot penetrate through the film to the Au substrate. Thus, the background is dominated by the tail of the C 1s edge from the droplet itself. To investigate the polarization dependence of the NEXAFS data, we collected spectra at normal incidence and 30° from grazing in p-polarization. The grazing incidence spectrum underwent a third normalization where the step from the pre-edge to the post-edge was matched to that measured at normal incidence. This is equivalent to a normalization per N atom. Other details regarding the interpretation of the data are described in the text below.

Results

Our experiments were designed to determine whether NEXAFS can be used to characterize two reactions that are widely employed for the covalent immobilization of biomolecules. The

EGFR peptide substrate Y1173 is used as a model biomolecule to investigate the reactions leading to its immobilization by measuring changes in the bonding configurations of nitrogen atoms involved in the immobilization reactions. As shown in Figure 1, the immobilization reactions involve three molecules: EG₄N, SSMCC, and Y1173. In each of these molecules, nitrogen atoms are found in various bonding configurations: nitrogen is present in EG₄N as a primary amine; in the SSMCC as an imide, and in the peptide in several different bonding states, with the most prevalent being that of the amide bond of the peptide backbone.

Figure 2 shows that the nitrogens in the various bonding states can be distinguished by their characteristic π^* and σ^* orbitals appearing in the N 1s NEXAFS spectra^{1, 9}. The technique is highly selective with respect to inequivalent nitrogen atoms. Figure 2A shows the NEXAFS spectra of EG₄N, SSMCC and Y1173 samples that were deposited onto the surface of a gold film by evaporation of solvent from solutions of the compounds (see Experimental Methods for details). We found that three transition energies can be distinguished as peaks in the NEXAFS spectra; the two transitions into π^* orbitals correspond to the imide bond of the SSMCC molecule (indicated as π_2^* in Figure 2A) and the amide bond of the peptide backbone (indicated as π_1^*). The absorption peak corresponding to the transition into a σ^* orbital is attributed to the amine of the EG₄N. Table 1 summarizes these results, and also provides a key to the various nitrogen atoms that are indicated in Figure 1C and 1D. Here we also note that the transition energies reported in Table 1 are consistent with previously published results involving NEXAFS of amino acids^{15, 16, 18, 20}. The central conclusion that emerges from the measurements in Figure 2A is that it is possible to use NEXAFS to distinguish between three nitrogen atoms present in three bonding states in our experimental system.

Next, we sought to determine whether the three states of bonding of the nitrogens atoms identified in Figure 2A can also be observed for the same molecules immobilized at monolayer coverage. In addition, we note that during reaction of the SSMCC with EG₄N, the primary amine (arrow 4 in Figure 1C) changes to an amide bond (arrow 2 in Figure 1C). Indeed, the associated change in the bonding of this nitrogen can be observed by comparing equivalent NEXAFS spectra in Figure 2A and 2B. Figure 2B shows spectra of the immobilized molecules during sequential steps of the immobilization procedure using a 100% EG₄N monolayer. In spectrum (iv), the SAM formed from EG₄N shows a peak at the energy of the σ^* orbital in Figure 2A, but due to the much smaller amount of material on the surface at monolayer coverage, the peak is far weaker. In addition to the main peak corresponding to the energy of the σ^* orbital, we noted the presence of a small shoulder corresponding to the energy of the π_1^* orbital from Figure 2A. We have not yet identified the origin of this peak but suspect radiation damage. Spectrum (v) shows that the reaction of the SSMCC with the EG₄N SAM leads to a new peak corresponding to the π_2^* transition of bulk SSMCC in Figure 2A. In addition, we find a small shoulder at the amide bond corresponding to the π_1^* orbital. As noted above, the primary amine of the EG₄N forms an amide bond during reaction with the SSMCC, and the appearance of the π_1^* feature provides confirmation of this reaction by NEXAFS. In order to further enhance the changes during the reaction of the SSMCC with the SAM we plot the difference spectrum in Figure 2C (upper spectrum): taking the difference enhances the shoulder at the transition energy π_1^* , corresponding to the formation of the amide bond upon reaction of EG₄N with the SSMCC.

The reaction of the SSMCC-activated EG₄N surface with the Y1173 peptide leads to the spectrum labeled (vi) in Figure 2B. A new peak appears at the energy corresponding to the π_1^* transition, in addition to peaks corresponding to π_2^* , and σ^* transitions. The difference spectrum resulting from the addition of the Y1173 peptide is shown in Figure 2C (lower spectrum). It clearly shows a strong π_1^* transition from the amide bonds along the backbone of the peptide. We also note that the reaction of the terminal thiol group of the oligopeptide

with the SSMCC does not change the bonding configuration of the nitrogen in either the peptide or immobilized SSMCC.

The reactions leading to the immobilization of the oligopeptide also affect the bonding configuration of the carbon atoms, as one can see in the corresponding C 1s NEXAFS spectra of Figure 3. First, we note that the normalized intensities are much larger than at the N 1s edge in Fig. 2, indicating that more carbon atoms than nitrogens are contained in the immobilized materials. The major observed peaks can be assigned to transitions into the π^* orbitals of C=O and C=C bonds, as indicated by vertical lines. The corresponding energies in Table 1 are consistent with literature reports^{15, 16, 18}. Inspection of Figure 3 reveals an increase in the C=O intensity with each step of the immobilization procedure (going towards the top spectra of the figure). This observation is consistent with the presence of C=O bonds in the imide group of the SSMCC and with additional C=O groups in the amide bonds of the Y1173 peptide. A significant $\pi^*_{\text{C=C}}$ transition occurs only upon immobilization of the oligopeptide. Indeed, carbon-carbon double bonds are only found in the tyrosine amino acid (benzene ring) of the oligopeptide. Thus the C 1s spectra reinforce the trends reported by the N 1s spectra and confirm a successful immobilization sequence.

The results in Figures 2 and 3 demonstrate that NEXAFS can be used to characterize two widely-employed reactions that involve the use of a heterobifunctional crosslinker to immobilize the Y1173 oligopeptide. We comment also that in the course of obtaining the above measurements, the results of NEXAFS measurements were helpful in improving the immobilization procedure. We found that the reaction of the SSMCC in PBS rather than water lead to higher immobilization densities of oligopeptides on the surface.

Next, we discuss the possible use of polarization-dependent NEXAFS to determine whether or not the peptides immobilized on these surfaces are oriented. We exploit the fact that the optical transition matrix element depends not only on the wave function of an orbital, but also on its orientation. Specifically, the transition intensity is maximized when the electric field vector $\mathbf{E}$ of the incident x-rays is parallel to the transition dipole moment for an atomic orbital, for example the N 2p orbital of a π^* bond along the backbone of a peptide. This phenomenon has been verified quantitatively¹⁸ in a small protein (RNase A). The transition intensity is proportional to the square of scalar product of $\mathbf{E}$ and the transition dipole moment, giving rise to a $\cos^2\theta$ distribution. The transition dipole moment is parallel to the axis of a σ bond and perpendicular to the axis of a π bond.

Figure 4A shows the polarization dependence of NEXAFS spectra obtained from the Y1173 oligopeptide immobilized by a SSMCC-activated EG₄N monolayer. Figure 4A shows total electron yield (TEY) spectra, while Figure 4B gives the simultaneously acquired fluorescence yield spectra (FY). Both data sets are qualitatively similar in their polarization dependence. Only the signal-to-background ratio is significantly higher in the FY spectra due to their better chemical selectivity from filtering the fluorescence photons. At the energy corresponding to the π^*_1 transition (the nitrogen in the peptide bond), the intensity of the TEY is larger at grazing incidence (30°) than at normal incidence. This result suggests that the immobilized oligopeptides adopt a preferred orientation relative to the surface. To obtain quantitative information from the spectra in Figure 4A, it is necessary to subtract the tail of the broad σ^* transition from the π^*_1 peak, which is indicated by dashed lines in Figure 4. This method of background subtraction eliminates artifacts due to a small drift of the signal (note that the peak of the TEY signal is less than 6% of the background from the Au substrate which is used for the normalization). Using this background subtraction method, we obtain a 32% increase of the TEY signal upon changing the angle of incidence of the x-rays from 90 to 30 degrees. A comparable increase of 38% is obtained from the more reliable FY signal in the bottom panel. The difference lies within the 10% error bar of the measurement. The key conclusion extracted

from these results is that it is possible to use NEXAFS to characterize the orientations of oligopeptides immobilized covalently to these surfaces, and that the Y1173 oligopeptide exhibits a preferred orientation on these surfaces. We have determined that it is possible to perform such polarization dependence measurements on peptide-decorated surfaces with densities of EG₄N as low as 1% by using FY measurements (see below). These results are reported in Figure S3 of the Supporting Information.

In principle, it should be possible to interpret polarization-dependent NEXAFS spectra to provide quantitative information about the orientation of the Y1173 peptide and its degree of order, as done for RNase A by Liu et al¹⁸. However, short oligopeptides lack well-defined secondary structure. Since there is no protein data bank (PDB) information about their structure, a quantitative interpretation will require the use of molecular dynamics simulations to provide insights into possible conformations that can be tested against the NEXAFS spectra. Such studies are underway and will be reported elsewhere. We note that an array of peptide bonds with a tilt of 33° from the normal for their unit vector $\vec{n}$ would exhibit the polarization dependence that is shown in Figure 4B (see Supporting Information for details of the assumptions and calculation). The Y1173 oligopeptide, however, is expected to possess peptide bonds with a distribution of orientations that are likely to differ substantially from 33°.

Many factors can be expected to influence the orientation of peptides on surfaces, including the density of peptides immobilized on the surface. Both inter-peptide and intra-peptide interactions need to be taken into account and measurements at low densities are needed to distinguish between them. In addition, the use of surface-based biological assays often necessitates the use of peptides at very low densities, requiring characterization of immobilized peptides at very low coverage. While TEY provides sufficient signal to observe a monolayer of immobilized peptides, we find the signal to be insufficient at sub-monolayer coverages. Measurements of the fluorescence yield (FY) with their improved signal-to-background ratio provide an avenue to characterize peptides on surfaces at lower densities, possibly low enough to be relevant to biological assays. Such data are shown in Figure S3 of the Supporting Information and in Figure 5.

Figure 5 shows the N1s absorption intensity of the π_1^* peak from the immobilized oligopeptide after subtraction of the background (see above for details), plotted as a function of the percent of EG₄N in the mixed SAM to which the oligopeptide was immobilized. Characteristic NEXAFS spectra associated with the data in Figure 5 are shown in Figure S3. The surfaces used to obtain the data points corresponding to 100% EG₄N are the same surfaces used to obtain spectra in Figures 2, 3, and 4. One can make several observations from Figure 5. First, the N1s absorption intensity generally increases with increasing concentration of EG₄N in the solution used to form mixed monolayer to which the peptide was immobilized (see below for additional discussion), thus providing support for using mixed SAMs to manipulate the density of oligopeptides on the surface. Second, a FY signal can be detected at concentrations of 1% EG₄N. If the peptides are evenly spaced at this concentration, there is approximately 15 Å between peptides, while the maximum length of a peptide is 20 Å. At this surface concentration one can expect that the interactions between oligopeptides on the surface would be relatively weak. Third, the data points in Figure 5 were each measured using three samples from the same batch. That reveals the reproducibility of the measurement at each surface composition (% EG₄N), which is generally very good. Although the overall trend observed in Figure 5 indicates that the N1s signal increases with EG₄N concentration, we note that the behavior at low EG₄N concentrations is not monotonic. A number of factors may lead to this unusual result, including the possibility of non-specific binding of the oligopeptide to the surface. Other factors may also contribute to this trend, such as differences in the kinetics of adsorption of the mixed monolayer that may result in different amounts of EG₄N immobilized on the surface. In addition, differences in inter and intra-peptide interactions at different densities may influence

the orientations of the peptides, and can contribute to apparent differences in the absorption intensity. While the exact cause of the trends in Figure 5 remains to be determined, the key conclusion that emerges from this study is that NEXAFS can be used to characterize peptides immobilized on surfaces over a wide range of surface densities. Such detailed characterization is rarely possible at the low densities of peptides shown in Figure 5.

Conclusions

In conclusion, we have demonstrated that NEXAFS can be used to characterize the sequential reactions involved in immobilization of an oligopeptide at a surface and the subsequent orientation of the biomolecule. Specifically, we have shown that NEXAFS can be used to distinguish the bonding states of carbon and nitrogen in amine-terminated SAMs, SSMCC, and Y1173 EGFR peptide substrates immobilized on surfaces. The differences in bonding configurations can be used to follow the reactions during each step of the immobilization procedure. We have also shown that it is possible to obtain polarization-dependent data that provide insights into the resulting orientation of the immobilized peptide. The measurements suggest that the Y1173 peptide has a preferential orientation, but a quantitative result requires more sophisticated modeling that takes disorder and the internal structure of the peptide into account. Finally, we have shown that it is possible to use NEXAFS to characterize the immobilization of the peptides at low surface densities relevant to biological assays, i.e. with little interaction between peptides. Overall, these results establish methods that can be used to optimize procedures for the immobilization of biomolecules on surfaces, and provide insights into the structural origins of the functional properties of biomolecules at surfaces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A–C: Schematic illustration of the three steps of the procedure used to immobilize the EGFR peptide substrate (Y1173) on a gold surface. **A:** Mixed monolayer of tetraethylene glycol (EG₄) and amine-terminated tetraethylene glycol (EG₄N). **B:** The heterobifunctional cross-linker SSMCC that has reacted with an EG₄N site via NHS chemistry. **C:** Y1173 immobilized via thiol chemistry to SSMCC. **D:** Structure of the 14 amino acid EGFR peptide substrate Y1173. Numbered arrows indicate different bonding configurations of nitrogen.

Figure 2.

N 1s NEXAFS spectra of (A) the bulk materials and (B) the three steps of the immobilization procedure. (C) shows the difference when adding the SSMCC linker to the EG₄N SAM (upper spectrum) and adding in addition the Y1173 peptide (lower spectrum). The peaks labeled π_1^* , π_2^* , and σ^* are assigned to specific N atoms labeled in Figure 1.

Figure 3.

C 1s NEXAFS spectra of the three steps of the immobilization of Y1173 (comparable to Fig. 2B). The spectrum of a nitrogen-free EG₄ SAM is included for comparison with the EG₄N SAM.

Figure 4. Polarization dependence of the N 1s NEXAFS spectra from immobilized Y1173 peptide at normal (90°) and grazing (30°) angle of incidence. The peptide bond at 401.4 eV shows significant polarization dependence, indicating a preferred orientation of the peptide on the surface. The dashed line indicates the background subtraction used to determine peak height quantitatively. (A) Polarization dependence using Total Electron Yield (TEY) detection. (B) Polarization dependence using Fluorescence Yield (FY) detection. Note the higher signal-to-background ratio in (B).

Figure 5. Intensities of the π_1^* peak in the N 1s NEXAFS spectrum obtained from spectra of immobilized oligopeptides, plotted as a function of the percent of EG₄N in the mixed SAM to which the oligopeptides were immobilized.

Table 1

Photon energies of individual NEXAFS peaks in the N 1s and C 1s NEXAFS spectra of surface immobilized SAMs, the heterobifunctional SSMCC crosslinker, and the Y1173 oligopeptide.

	Assignment	Energy	Bonding Configuration	Occurrence
Nitrogen	π_1^*	401.4 ^{15, 16, 18, 20}	Amide Bond (arrows 1,2)	Peptide backbone NHS reacted SSMCC
	π_2^*	402.4	Imide Bond (arrow 3)	SSMCC
	σ^*	405.3 ^{15, 16, 18, 20}	Primary Amine (arrow 4)	EG ₄ N Y1173 Peptide, N terminus
Carbon	$\pi^*_{C=C}$	285.0 ^{16, 18}	C=C	Tyr residue of peptide
	$\pi^*_{C=O}$	288.1 ^{15, 18}	C=O	SSMCC Peptide backbone and certain amino acids