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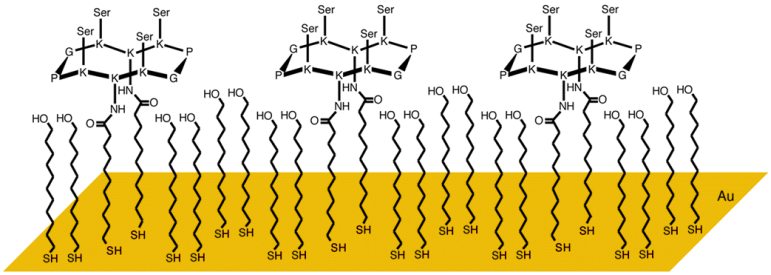

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Orientation Modulation of a Synthetic Polypeptide in Self-Assembled Monolayers: A TOF-SIMS Study

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Abstract: Structure and orientation of molecules are key properties of functionalized surfaces. Using time-of-flight secondary ion mass spectrometry (TOF-SIMS), here we investigate how to modulate these parameters upon the immobilization process varying the conditions of self-assembly. The molecule of interest, a template-assembled synthetic protein (TASP), consists of a central peptide ring with orthogonally arranged residues. Thioalkane chains allow the directed self-assembly of the molecule on a gold surface; four serine residues on the opposite side of the ring can be used as anchoring sites for various functional sensing molecules. The TASP conformation and its orientation in self-assembled monolayers (SAMs) play a central role for the accessibility of these serine residues. To study the influence of the self-assembly conditions, two series of samples were prepared. Pure TASP monolayers of different surface densities are compared to mixed TASP/alkanethiol monolayers prepared by sequential adsorption varying sequence and particular incubation times as well as by coadsorption modifying incubation times and TASP/alkanethiol mass ratios. Switching the TASP orientation from a state where the molecules are lying flat on the surface to an upright orientation turned out to be possible by inserting the TASP into a preformed alkanethiol monolayer of an appropriate surface density. This study demonstrates that TOF-SIMS is an excellent tool not only to investigate the surface composition, but also the molecular structure of functionalized surfaces.

Introduction

Self-assembled monolayers (SAMs) on solid supports are widely used to create specific physical and chemical surface properties.[1,2] Because of their capabilities to modulate interfacial properties such as wettability,[1] adhesion,[3] tribology,[4] and biocompatibility,[5] such molecular thin films are suited for the design and production of smart functional materials. Surfaces, functionalized by SAMs, are very often prepared by sulfur-bearing molecules such as thioalkanes, thiolipids, modified oligonucleotides, polypeptides, and proteins or combinations of them.[6−8] Such surfaces are of increasing importance in detecting and screening compounds by surface sensitive techniques in the field of chemical and biological analytics.[9,10] Novel possibilities for micropatterning surfaces[10−14] and for producing functionalized cantilevers[15] open the door for the creation of multiarray micro- and nanoscale sensors suitable for high-throughput screening in pharmaceutical, food, and environmental applications.

The formation of surfaces comprising active molecular-sized assemblies with predictable functions requires the control of the molecular architecture of the SAMs and thus the understanding of the fundamental molecular self-assembly processes on the particular surfaces. In a series of papers, we and others have investigated SAMs of synthetic polypeptides with designed properties and functions.[16−21]

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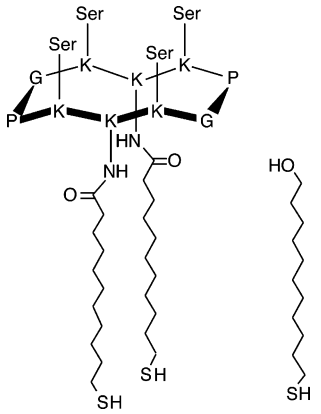

Figure 1. Molecular structures of TASP and hydroxyundecanethiol. The amino acid code is K, lysine; P, proline; G, glycine; Ser, serine.

Here we report on the self-assembly of a template-assembled synthetic polypeptide (TASP)[22−25] on gold surfaces, the modulation of the orientation of the TASP, and the detection of the structural changes by time-of-flight secondary ion mass spectrometry (TOF-SIMS). The TASP comprises a central cyclic peptide template with attachment sites on opposite faces of the cycle (Figure 1). Two long chain carboxyalkanethiols are covalently attached via peptide bonding to the lower side of the template to allow directed self-assembly of the TASP on the gold surface via Au−S bond formation. Four serine residues on the upper side of the template are potential anchoring sites for the attachment of functional sensing molecules to detect ions, oligonucleotides, or proteins. We demonstrated recently that such a TASP can be immobilized on gold surfaces, subsequently functionalized by antigens, and finally used to measure antibody binding by surface plasmon resonance.[25]

For the discussion of possible orientations of the surface-immobilized TASP, we refer to Figure 2. In pure TASP SAMs, we expect that the molecules are lying flat on the surface due to strong peptide gold interactions (Figure 2A). In contrast, in a mixed SAM of TASP and alkanethiols, the latter are expected to covalently bind to the surface via Au−S bond formation and to intercalate between the hydrocarbon chains of the TASP and thereby force the TASP hydrocarbons to a reduced tilt angle or even to a perpendicular chain orientation with respect to the gold surface. Moreover, incubation times and sequence of thiol and TASP immobilization are supposed to play a role in the layer formation. A modulation of the hydrocarbon chain orientation might in turn also induce changes of the peptide template ring orientation and thus changes of the accessibility of the functional serine groups on the peptide ring (Figure 2B). Furthermore, if the TASP is adapting an upright orientation within a thiol matrix, its peptide ring cannot get into contact with the gold surface, thus avoiding a possible denaturation due to strong peptide gold interactions.[26]

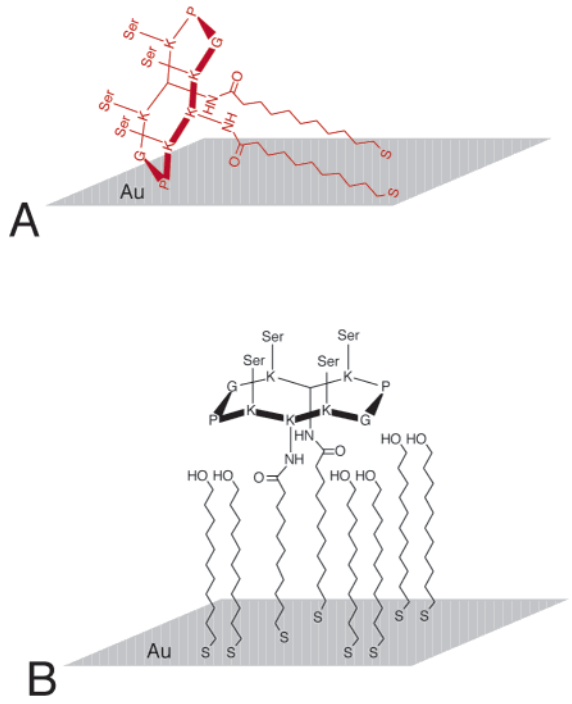

Figure 2. Schematic views of the TASP molecule. The TASP is supposed to be attached to the surface via gold sulfur bonds of the SH groups at the termini of the hydrocarbon chains; disulfide formation may occur. (A) The molecule lying flat on the surface. In addition to the Au−S interactions, there is direct contact between the peptide ring and the gold surface. Possible bonding configurations include also amino acid/gold interactions, for example, the Au−proline interaction discussed in detail. (B) The molecule in an upright orientation within a thiol matrix. The peptide ring is decoupled from the gold surface by a hydrocarbon layer. Drawing is schematic, because the exact tilt angle of the TASP in this orientation is not known.

To study this influence of the self-assembly conditions on the TASP molecular orientation, two series of samples were used. Pure peptide layers of different surface densities were prepared by varying the incubation time of the gold surface in the TASP solution. In a second approach, the TASP molecules were integrated into a SAM matrix of hydroxyundecanethiol (Figure 1). This alkanethiol is known to form densely packed SAMs on gold surfaces.[27] Its hydrocarbon chain is as long as the alkanethiol spacers of the TASP molecule, and it has a hydrophilic headgroup that has a low tendency to denature the TASP's hydrophilic cyclic peptide. The mixed TASP/alkanethiol SAMs were prepared (i) by sequential adsorption of TASP and thiol, varying the sequence and the particular incubation times, as well as (ii) by coadsorption, applying different TASP/thiol mass ratios and incubation times.

All SAMs were analyzed by TOF-SIMS. This method allows the direct chemical analysis of a solid surface.[28] The surface is bombarded with a pulsed primary ion beam inducing the desorption of particles. The mass(-to-charge ratio) of their charged parts, the so-called secondary ions (SIs), is determined by measuring the time-of-flight they need for a distance of a certain length. Because of the so-called matrix effect, the fact that the desorption probability of particles and the formation probability of particular SI strongly depends on its surface structure, orientation and molecular surroundings, not only the

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surface composition but also the orientation and steric arrangement of molecules can be analyzed.[29] On the basis of this effect, coexisting lipid phases have been visualized,[30] and the surface exposed groups of different lipid layers[31] have been determined, just to give some examples. Here we use TOF-SIMS for the analysis of peptides and their orientation on surfaces as well as for the control of the stepwise production of functionalized surfaces.

Experimental Section

Materials. The TASP was synthesized by a combined solid-phase/solution strategy as described elsewhere;[32] the hydroxyundecanethiol was synthesized following standard procedures.[33] Water was purified to a final resistivity ≥18 MOhm cm using a MilliQ purification system. MeOH and EtOH were purchased from Fluka in the highest quality available.

Sample Preparation. Glass slides were thouroughly cleaned by ultrasonication first in aqueous detergent solution (Hellmanex, Hellma, Müllheim, Germany), and subsequently in water. On these slides, chromium adhesion layers of 3 nm thickness followed by gold layers of a thickness of 100 nm were formed by vapor deposition at a pressure of 5×10^{-6} mbar. After being cooled to room temperature in the vacuum chamber of the evaporator, the samples were immediately transferred into the self-assembly solutions.

The TASP was self-assembled from methanolic solution, the alkanethiol from a water/ethanol mixture (v:v = 1:1). Both the thiol and the TASP concentration were 1 mg/mL. For TASP/thiol coadsorption, the pure solutions were mixed in appropriate amounts. After self-assembly, the samples were thouroughly rinsed with the respective solvent, dried in a stream of nitrogen, and immediately introduced into the TOF-SIMS setup.

TOF-SIMS Spectra. Mass spectra were recorded using a homemade apparatus at the GSF Munich described in detail elsewhere.[34] For the spectra presented in this study, we used SF_5^+ projectiles with a primary voltage of 30 kV and a dc primary ion (PI) current of about 1.3 nA. The spot size was $300 \times 400\ \mu m^2$ at an apparent impact angle of 60°. However, the actual exact angle and energy of impact depend on the target bias and polarity. The target was typically biased with ±4.5 keV. The SF_5^+ beam was chopped at 20 kHz, with a pulse length of 7 ns; 20×10^6 primary pulses were integrated to get statistically meaningful data. Hence, the maximum PI fluence was less than 1.5×10^{12} PI/cm^2, meaning that the measurements were carried out within the static SIMS regime, that is, under practically nondestroying conditions. With a time resolution of the time-to-digital converter of about 0.5 ns, the mass resolution M/dM (dM = full width at half-height (fwhh)) was about 500 at a mass of about 1000 atomic mass units (u), partly limited by the pulse width of the PIs. All spectra were standardized on a PI dose density of 1×10^{12} PI/cm^2. Characteristic peak intensities could be quantitatively reproduced by repeatedly recording spectra of the same sample (less than 10% deviation). Spectra of negative and postive SIs were recorded routinely, but only spectra of negative SIs were considered, as the spectra of positive SI do not provide any further information.

Table 1. Different Preparation Strategies and Corresponding Relative Intensities for Five Selected SIs (Abbreviations: P, TASP; T, Hydroxyundecanethiol; IT, Incubation Time; P/T, TASP/Thiol Mass Ratio)

preparation		relative intensities of secondary ions				
preparation mode	IT	OCN^-	Au(proline)$^-$	Au_3^-	Au_2-thiol$^-$	TASP ring$^-$
Au		7171	217	3922	44	9
thiol only	20′	21 570	425	2970	2893	17
TASP only	30″	143 250	4454	2457	104	27
	5′	213 310	7023	3802	183	25
	30′	143 390	4861	2091	131	22
	60′	181 910	5602	2277	106	20
	240′	128 920	2642	1300	140	18
(1) TASP, (2) thiol	5′, 5′	297 580	4629	2383	371	29
(IT_P, IT_T)	5′, 20′	98 600	2412	1151	277	24
	5′, 60′	284 380	2774	2478	724	43
	10′, 20′	80 978	2022	863	225	12
	20′, 5′	91 724	2019	784	147	24
	120′, 5′	66 227	1649	658	104	17
	120′, 60′	112 090	2944	1112	131	23
coadsorption	1:2; 25′	35 434	443	2074	2415	131
(P/T, IT)	1:2; 60′	8611	259	2945	236	10
	1:1; 25′	8475	436	2621	436	11
(1) thiol, (2) peptide	5′, 5′	11 542	296	964	829	37
(IT_T, IT_P)	5′, 20′	21 111	303	2236	520	15
	20′, 3′	7489	487	2267	2898	157
	20′, 5′	15 460	401	2332	4317	370
	20′, 10′	20 167	331	1530	2740	282
	20′, 20′	12 639	269	3395	340	14
	30′, 5′	18 430	410	1297	2093	94

Results and Discussion

Overview. TASP SAMs were formed applying different preparation protocols: Pure TASP layers of different surface densities were prepared by varying the incubation time of the gold surface in the TASP solution between 30 s and 240 min. Mixed TASP/hydroxyundecanethiol SAMs were prepared (i) by sequential adsorption of TASP and thiol, varying the sequence and the incubation times, as well as (ii) by coadsorption, applying different TASP/thiol mass ratios and incubation times. Pure gold surfaces and pure hydroxyundecanethiol SAMs served as references. Preparation details as well as the TOF-SIMS results are summarized in Table 1. Partial spectra of two extreme cases of resulting surface orientations are shown in Figure 3. The corresponding partial spectra of negative SIs for all other preparations are published in the Supporting Information.

To evaluate the influence of the SA conditions on the TOF-SIMS fragmentation pattern of the SAM molecules, from the wealth of SIs, we chose five representative examples for discussion. The first selected SI is the peptide bond fragment OCN^- (42 atomic mass units, u), which is typical for peptides in general.[35] Furthermore, we have chosen two SIs that elucidate the orientation of the TASP molecule on the surface: The SI Au(proline)$^-$ (Au(C_5NH_6), 277 u) is attributed to the amino acid proline attached to gold. This TASP-SI, one example of the SIs representing an amino acid attached to gold, can only appear if the molecule is not only attached to the gold surface via the SH groups at the hydrocarbon termini, but also all or a part of the cyclic peptide contacts the gold surface; that is, the

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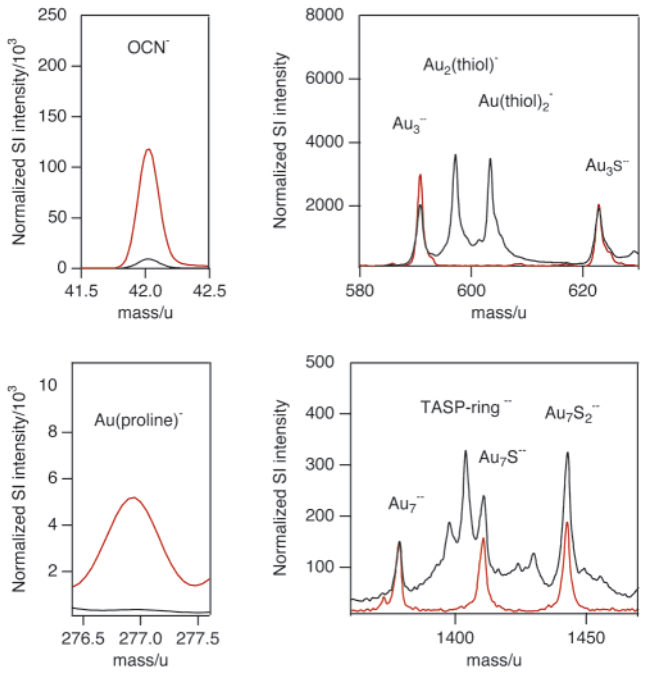

Figure 3. Partial TOF−SIMS spectra for two extreme cases of preparation: In red, data obtained for a pure TASP layer (IT_P = 10 min) are shown. Here the TASP molecules are lying flat on the surface. The black curves represent data obtained for a mixed peptide/thiol SAM prepared by sequential adsorption of thiol (IT_T 20 min) and subsequently TASP (IT_P = 5 min). Here the TASP molecules adapt an upright orientation. The following SIs not mentioned in the text are visible in the spectra: the gold cluster Au_7^- (1379 u), the gold/sulfur clusters Au_3^-S (623 u), Au_7^-S (1411 u), and $Au_7^-S_2$ (1443 u), as well as Au-thiol$_2^-$ (603 u) and several high mass SIs built from the TASP-ring$^-$ by cleavage or attachment of NH, OH, etc. Schematic views of the molecular structure and orientation obtained for the two different preparation modes are shown in the corresponding colors in Figure 2.

TASP molecule is lying flat on the surface (Figure 2A). If the TASP molecule is in an upright orientation (Figure 2B), this SI can hardly be formed. Instead, the SI representing the TASP peptide ring without thiol spacers (TASP ring$^-$, 1404 u) is supposed to be dominating. If the molecule lies flat on the surface, it is subject to strong fragmentation due to the large surface area per molecule and strong TASP/surface interactions: High mass TASP-SIs are not expected. Because we do not know the exact tilt angle of the TASP in the two different orientations, for simplicity we name them "upright" and "flat" in the following. For none of the preparations does the molecular ion of the TASP (1826 u) occur in the spectrum, even for the preparations in which the molecule is found to be in the upright orientation. Apparently, the molecules are always cleaved above the attached alkanethiol chains. SIs of gold clusters are released from the support. Spectra of alkanethiols on gold are generally characterized by rather unspecific SIs of the hydrocarbon chain as well as gold−sulfur clusters, and SIs representing the headgroup and the molecular ion.[36] We selected the SI Au_3^- (591 u) as representative example. As a typical thiol SI, we show Au_2-thiol$^-$ (597 u).

Pure Gold Surface and Pure Hydroxyundecanethiol SAM on Gold. The pure gold surface was measured immediately after its vapor deposition without further treatment. As expected, the intensity of the Au_3^- SI is high, whereas signals corresponding to TASP- and thiol-SIs are not present. Only the OCN$^-$ SI occurs in a considerable intensity, probably due to contamination of the gold surface. The pure alkanethiol SAM on gold was prepared by 20 min incubation in a thiol solution. A high intensity of the thiol SI, Au_2-thiol$^-$, the absence of most TASP-SI, as well as a pronounced decrease in intensity of the gold SI prove the presence of the alkanethiol layer on gold. The presence of OCN$^-$ SI we trace again back to contaminations of either the TASP or airborne species.

Pure TASP SAMs. For the preparation of pure TASP SAMs of different surface molecular densities, five different incubation times were tested: 30 s, and 5, 30, 60, 240 min. For all incubation times, the TOF-SIMS spectra are typical for TASP layers on gold: OCN$^-$-SI represents the peptide bondings. The intensity of gold SIs is strongly reduced due to the presence of the SAM. The presence of the SI Au(proline)$^-$ and the absence of high mass TASP-SI indicate that the TASP's cyclic peptide is in contact with the gold surface; that is, the molecule adopts the flat orientation. Varying the incubation time does not have any influence on the orientation. The intensity of the TASP-SI OCN$^-$ and Au(proline)$^-$ is maximal at 5 min incubation time. Otherwise, no clear trend is visible. This behavior might be due to a complex time course of the adsorption process, such as a rapid initial adsorption followed by a slow reorientation or packing process. Furthermore, a matrix effect might occur: The desorption probability is likely to be inversely proportional to the layer density.

First TASP, then Thiol Adsorption. Seven different incubation time protocols were tested (for details, see Table 1). The TOF-SIMS spectra of all preparation resemble very much the ones obtained for pure TASP SAMs discussed before: For all different combinations of incubation times, Au(proline)$^-$ SIs are dominating. Obviously, once the TASP SAM is formed, the thiol cannot enter this dense peptide film, and a thiol matrix cannot be formed. Only for a short TASP incubation time (5 min) combined with a long thiol incubation time (60 min) are thiol SIs present in low amounts in the TOF-SIMS spectrum.

TASP and Thiol Coadsorption. Here, the surface composition and hence the molecular structure of the SAM depend on the composition of the incubation solution and the incubation time applied for the self-assembly process. For a relatively high TASP portion in solution (mass ratio 1:1; incubation time 25 min), the TASP molecule is lying flat, and thiol adsorption is hindered: Au(proline)$^-$ SIs are detected, whereas thiol SIs do not occur. For a higher thiol portion (m:m = 2:1) and the same relatively short incubation time, however, the thiol is present in high surface concentration. We conclude that the TASP lies no longer flat on the surface but adapts an upright orientation. This is indicated by the presence of the TASP ring$^-$ SIs and the absence of Au(proline)$^-$ SIs as well as a pronounced decrease in the OCN$^-$ SI intensity due to less fragmentation. Finally, for the same thiol portion combined with a longer incubation time (60 min), again only little thiol is present on the surface, and the TASP molecule is no longer in an upright orientation. Probably the thiol is displaced by the peptide during longer incubation times. Such thiol displacement, in the present case favored by the two attachment points of the TASP, has been reported previously.[37]

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First Thiol, then TASP Adsorption. Seven different incubation time protocols were tested (for details, see Table 1). Applying a medium hydroxyundecanethiol incubation time (IT_T) combined with a short TASP molecule incubation time (IT_P) results in TASP molecules in an upright orientation embedded in a thiol matrix as indicated by the TOF-SIMS spectra showing high intensities of thiol and TASP ring$^-$ SIs. For $IT_T = 20$ min followed by $IT_P = 5$ min, the intensity of the TASP ring$^-$ is maximal. However, for shorter IT_T's, the corresponding TOF-SIMS spectra show Au(proline)$^-$ SIs and a lower intensity of the thiol SI. This indicates that the TASP molecules are lying flat; presumably the thiol matrix is not sufficiently dense and well-ordered to force the TASP into an upright orientation. For medium IT_T's and medium IT_P's, the TASP lies also flat on the gold surface. Because of the higher IT_P, probably alkanethiol displacement takes place and the thiol matrix density is decreased below a critical density necessary to induce an upright orientation of the TASP. In contrary, for high IT_T's the thiol matrix seems to be too dense to allow the insertion of TASP molecules into the alkanethiol SAM.

Conclusions

Static TOF-SIMS proved an excellent tool to control the stepwise preparation of functionalized surfaces: It is not only very well suited for the analysis of the surface composition of SAM but also for investigating their molecular structure and orientation. This is not restricted to low mass substances: both presence and molecular orientation of the TASP molecule can be monitored. To the best of our knowledge, this is the first time that the surface orientation of a molecule of this size and complexity could be determined using TOF-SIMS. Part of this success we trace back to the use of SF_5 primary ions. The application of these polyatomic ions is well-known to strongly enhance the yield of negatively charged SIs, in particular of those of high mass.[34] Moreover, all samples were prepared on gold, which has previously been shown to be an excellent surface material for the investigation of soft biological samples by TOF-SIMS, because the molecules can be gently desorbed together with surface gold clusters.[30]

Using TOF-SIMS, we could study the influence of the self-assembly protocols on the TASP molecular surface orientation. Switching the TASP orientation from a state where the molecules are lying flat on the surface to a second state where they are immobilized in an upright orientation turned out to be possible by inserting the TASP molecules into an alkanethiol matrix of an appropriate molecular density. This procedure might be a generic approach to modify the molecular orientation and thus switch functional properties of large molecules on surfaces.

TOF-SIMS turned out to be unique for observing these changes of the molecular layer structure. Fourier transform infrared spectroscopy (FTIR) and surface plasmon resonance (SPR) or ellipsometry, in principle suited to deliver information on surface structure and orientation, fail in the present case.

From NMR and circular dichroism experiments and restrained molecular dynamics simulations, it was concluded that the TASP structure comprises two distorted antiparallel β-sheet strands connected by two type II β-turns.[38] A β-strand contribution in the Amide I and II region in our previously published FTIR spectrum of a pure TASP layer is in agreement with the conservation of this structure on gold.[25] However, the opposite in and out of plane orientations of the N−H and C═O bonds do not allow any unequivocal determination of the whole template orientation on the surface by FTIR spectroscopy.

SPR or ellipsometry in general deliver information on the average thickness or mass per surface area of a molecular film. For a pure TASP layer, the surface area per molecule has been found to be 240 Å^2, corresponding to a molecule lying flat on the surface.[25] This finding is in agreement with our TOF-SIMS results. For mixed TASP/thiol layers, however, it is no longer possible to attribute unequivocally a molecular surface area to one of the components from SPR data. Without prior knowledge of the molecular organization, the different contributions to the optical thickness cannot be separated, because the system is underdetermined.

In view of the above-discussed limitations and uncertainties of complementary surface sensitive techniques, the present study demonstrates that TOF-SIMS is ideally suited for the determination of peptide orientations on surfaces delivering important new information which would not be available from other techniques.

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Supporting Information Available: Partial spectra of negative SIs for the preparations not shown in Figure 3 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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