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Functional Molecular Thin Films: Topological Templates for the Chemoselective Ligation of Antigenic Peptides to Self-Assembled Monolayers**

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The study of molecular thin films for use in the controlled design of interfacial properties[1] that have important implications in many different fields ranging from research on friction, lubrication and wetting,[2] development of micro- and nanoscale devices[3] to biocompatible surfaces, has attracted considerable interest.[4] Most of the work has concentrated on self-assembled monolayers (SAMs), in particular on the self-assembly of sulfur-bearing molecules such as thioalkanes[5] and lipids[6] on the surface of gold, or suitable silanes to hydroxylated surfaces (usually silicon oxide or glass).[7] Self-assembled molecular thin films that comprise biopolymers are most interesting for the development of novel analytical techniques.[8] Several novel micropatterning techniques have opened the possibility for the creation of multiarray sensor devices on the level of SAMs.[9] In particular in the latter case, a generally applicable method for the design and synthesis of complex functional peptides suitable for the integration into SAMs is still a demanding task. We have shown recently that tethering template-assembled synthetic proteins (TASP) that expose metal or antigenic binding sites to SAMs results in highly sensitive and selective functional surfaces.[10, 11] Here we elaborate novel strategies based on the template concept for the regioselective functionalization of SAMs on gold surfaces. In particular, regioselectively adressable functional templates (RAFT) that feature differentially reactive, spatially distinguishable domains in combination with chemoselective ligation procedures are used for the covalent attachment of antigenic peptides to SAMs. The formation of the SAM, its functionalization by several consecutive surface chemistry reactions, and the final binding of antibodies to the tethered TASP, were monitored and controlled by surface plasmon resonance (SPR), time-of-flight secondary-ion mass spectrometry (TOF-SIMS), and Fourier transform infrared (FTIR) spectroscopy. These methods are capable of measuring molecular reactions on gold surfaces: SPR delivers direct and continuous information on the mean concentration of the molecules on the surface during the formation of monomolecular films, TOF-SIMS allows the direct analysis of the chemical composition of SAMs, FTIR spectra carry information on both the presence of specific functional groups and the conformation of the molecules on the gold surface. The FTIR spectra were obtained by a recently developed attenuated total reflection (ATR) technique where the ATR crystal was covered by a gold film that is thin enough to be transparent for the infrared light but thick enough to serve as a continuous gold surface for the formation of SAMs by thioalkanes.[12]

As a representative example of this general approach of functionalizing SAMs we covalently coupled a derivative of the antigenic $(NANP)_3$ peptide to a SAM of topological templates. This peptide was chosen because of its relevance to the immune response against malaria parasites; in another context it has been immobilised on gold surfaces by thioalkane linkers.[13] As the RAFT molecule we used a cyclic peptide of the sequence c[(K(Boc)K(Boc)PGK(Alloc))$_2$] **1**, which features orthogonally protected attachment sites on opposite faces of the cycle.[14] Carboxythioalkanes were attached in bulk solution to the lower face of the deprotected K(Alloc) side chains of the template **1**, followed by the coupling of serines to the remaining deprotected K(Boc) sites of the serine groups. Subsequently, the RAFT molecule **2** was self-assembled on a gold surface through the thioalkane linkers. The hydroxyl groups on the four serine side chains that are on the upper face of the template were oxidized to aldehyde functions (Figure 1). Finally, the aminooxyacetyl-containing antigenic peptide **4** was ligated to the surface-

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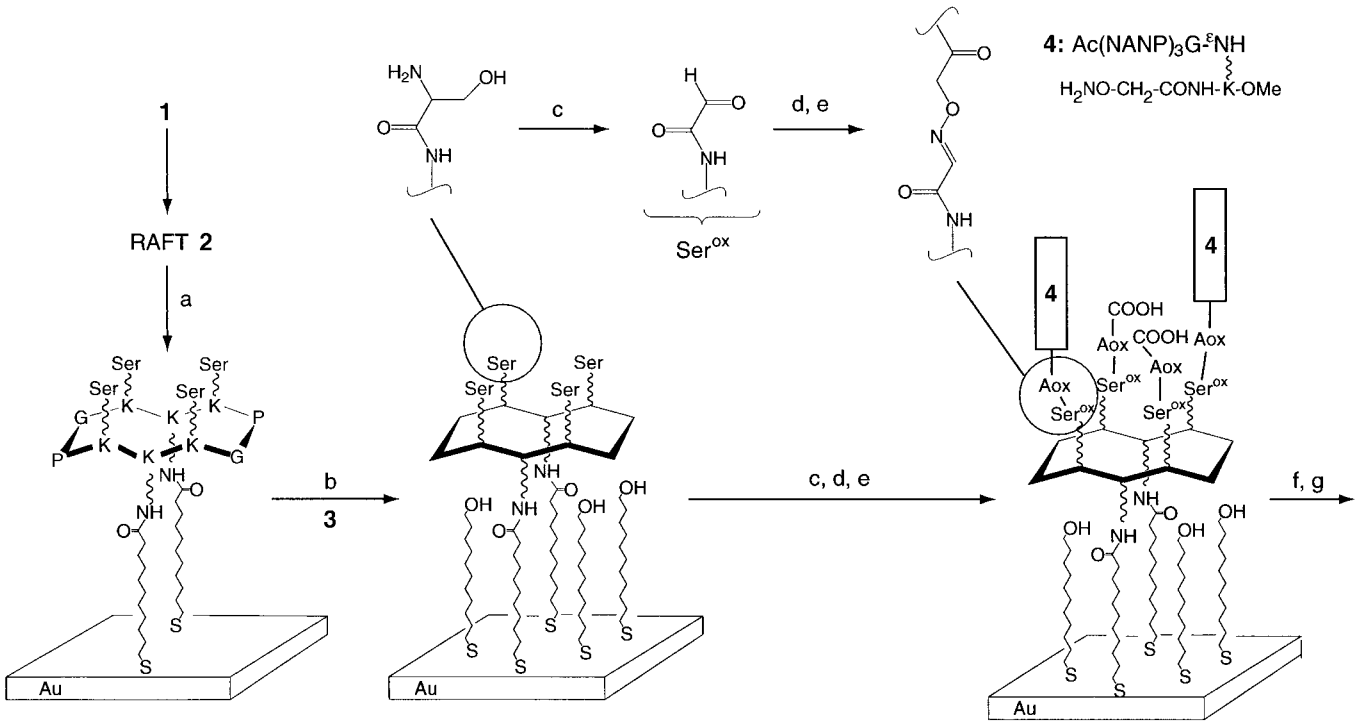

Figure 1. Individual reaction steps of the TASP synthesis on a gold surface: a) Self-assembly of **2** on the surface, b) blocking of the remaining bare gold surfaces (gaps and defects in the SAM) by 11-sulfanylundecanol (**3**), c) oxidation of N-terminal serine residues, d) chemoselective ligation of peptide **4** to the surface, e) blocking of the remaining terminal aldehyde groups. The thus functionalized gold surface comprises receptor sites for the selective binding of the monoclonal antibody (f), which can finally be displaced from the surface under controlled conditions by the application of excess free antigen Ac-(NANP)$_3$G-OH to the bulk solution (g). Steps (a) – (f) were observed by ATR – FTIR spectroscopy (Figure 2), steps (c) and (e) by TOF – SIMS (Figure 3), steps (a) – (g) by SPR measurements (Figure 4). The amino acid residues are given in one letter code except for serine so that it can be distinguished from the terminal sulfur atom attached to the gold surface.

assembled RAFT by formation of oxime bonds.[15] The resulting monomolecular film on the gold surface contained functionalities for the selective and reversible binding of the monoclonal antibody Sp3E9, which is directed against the NANP peptide. The whole sequence of surface reactions comprises seven individual steps:

a) A SAM was formed by adsorption of **2** from a methanolic solution onto a gold surface. A resonance angle shift of $\Delta\Theta = 0.37^\circ$ was observed by SPR, which corresponds to a mean surface area of 240 Å^2 for an individual molecule of **2**. This value is somewhat higher than expected from the template geometry, possibly as a consequence of the bulkiness of the carboxythioalkane chains and serine residues attached to the template. The FTIR spectrum of **2** on gold shows the amide I and II bands at similar wavenumbers and with similar shapes (Figure 2, (a) – Au) as their counterparts in the bulk material on ATR plates.

b) The remaining bare gold surfaces that result from gaps and defects in the SAM of **2** were filled by adsorption of 11-sulfanylundecanol (**3**) from a solution in ethanol/water (1/1). This modification resulted in the appearance of an OH-bending band at 1640 cm^{-1} in the FTIR spectrum (Figure 2, (b) – (a)). The SPR resonance angle was shifted by $\Delta\Theta = 0.18^\circ$.

c) The serine-terminated template monolayers were oxidized by a solution of $NaIO_4$ in citric acid/sodium phosphate buffer (pH 3.3) for 20 min. As a result of the small change of the molecular mass this reaction was not detectable by SPR;

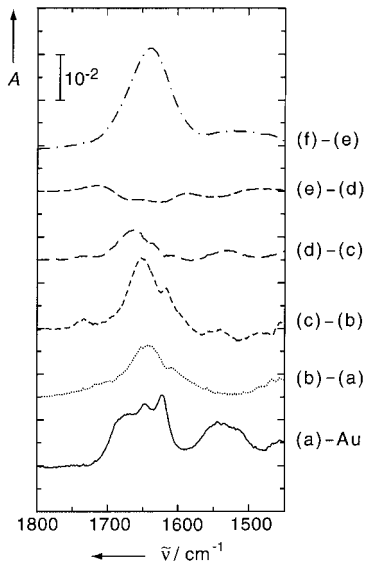

Figure 2. ATR – FTIR difference spectra of the monomolecular layers at gold surfaces for the consecutive reaction steps (a) to (f) (see text and Figure 1).

however, a new band at 1660 cm^{-1} in the FTIR spectrum could be attributed to the formation of aldehyde groups in the template (Figure 2, (c) – (b)). This was confirmed in the TOF-SIMS spectrum (Figure 3A) by a signal at *m/z* 1702, which corresponds to the molecular ion of the template oxidized at all four serine residues.

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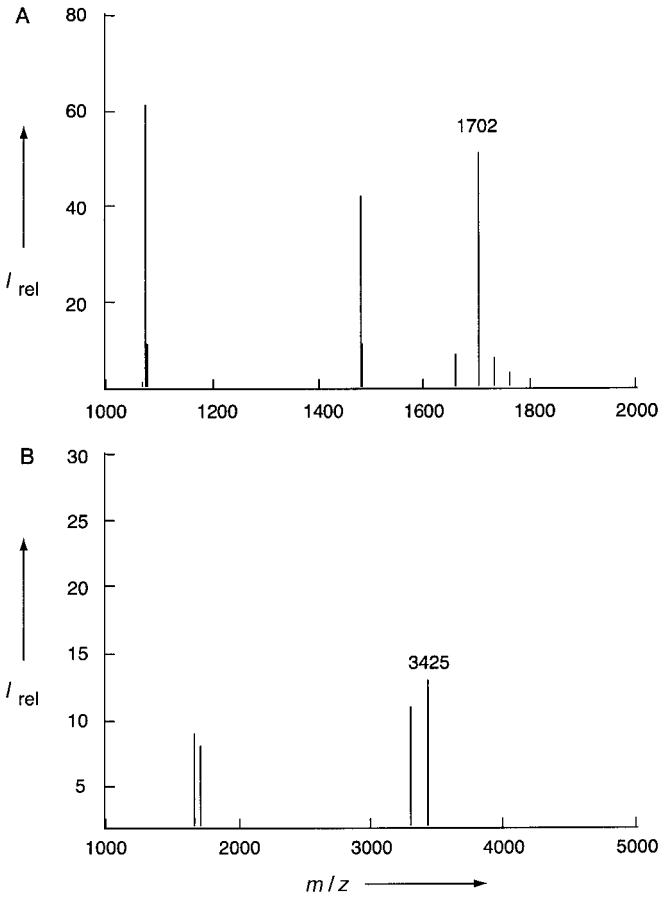

Figure 3. TOF–SIMS spectra of negative secondary ions recorded A) after step (c) and B) after step (e) of the reaction scheme shown in Figure 1.

d) The coupling of peptide **4** to the modified RAFT molecule was performed overnight at room temperature, again in the citric acid/sodium phosphate buffer. Two new bands appeared at 1657 and 1528 cm^{-1} in the FTIR spectrum (Figure 2, (d)–(c)). These bands correspond to the amide I and II bands of the peptide **4**, which were observed at similar positions in the bulk peptide sample. An SPR resonance angle shift of $\Delta\Theta = 0.50^\circ$ was finally obtained; this corresponds to the condensation of about two molecules of **4** (mean surface area of 115 $Å^2$) to each of the adsorbed RAFT molecules. Incomplete oxidation of the serine residues on the template can be excluded as the origin of this partial saturation of the attachment sites on **2** by **4**, as a prolonged oxidation (step c) did not result in a higher surface density of **4** as measured by SPR. In accordance with earlier observations,[13b] peptide **4** seems to be sterically saturated at the surface of the monomolecular film. In contrast to the situation at the SAM surface, all ligation sites of **2** can be selectively functionalized in solution.

e) The unchanged terminal aldehyde functions at the monolayer were modified by treatment with excess aminooxyacetic acid. This step affected neither the SPR nor the FTIR spectra (Figure 2, (e)–(d)), but the corresponding reaction products were clearly visible in the TOF–SIMS spectrum (Figure 3B): The peak at *m/z* 425 corresponds to a RAFT molecule with one molecule of **4** andthree serine residues modified by aminooxyacetic acid. The lack of a peak corresponding to RAFT molecules with two or more peptide molecules per template might be a result of the low desorption and transmission probability of this extremely high mass secondary ion.

f) The activity of the tethered TASP layer was assayed by antibody binding experiments: the functionalized monolayer at the gold surface was incubated with a 4.7×10^{-7} M solution of the antibody in PBS buffer. This produced an intense amide I band at 1640 cm^{-1} (Figure 2, (f)–(e)) in quantitative agreement with the FTIR spectrum of the bulk antibody sample. Concomitant SPR measurements showed a shift in the resonance angle of $\Delta\Theta = 1.10^\circ$ (Figure 4).

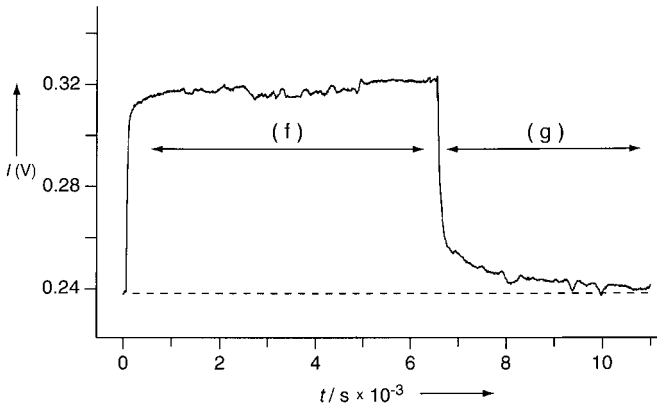

Figure 4. On-line SPR measurement of the antibody binding to the tethered TASP on the gold surface (step (f) in Figure 1), and subsequent antibody displacement upon incubation of the monolayer with excess free antigen in the bulk solution (step (g)). I(V) is the electrical signal of the photodiode, which is proportional to the reflected light intensity.

g) Addition of a suitably high concentration (10^{-4} M) of the antigenic peptide Ac-$(NANP)_3$G-OH to the bulk aqueous buffer solution displaced the monoclonal antibody from the monolayer surface as indicated by the SPR resonance angle shift of $\Delta\Theta = -1.03^\circ$ (Figure 4). This demonstrates the reversibility of the antibody binding to the functionalized surface.

In conclusion, chemical ligation methods in combination with regioselective addressable templates have allowed the preparation of well defined, complex monomolecular films tethered to gold surfaces. The supramolecular monolayers exposed artificial recognition sites, here for monoclonal antibodies. SPR, FTIR spectroscopies, and TOF–SIMS were ideally suited surface sensitive techniques to enable the essential control of the individual reaction steps. The concept presented here for the functionalization of gold surfaces may be easily applied to other supporting materials such as glass(like) surfaces, it is certainly not limited to peptides but can be equally well extended to proteins and other biopolymers such as polynucleotides (DNA or RNA). This approach can be extended, in conjunction with methodologies, for chemoselective ligation and orthogonal protection techniques[16] to the construction of multifunctional surfaces with interesting potential in bioanalytics.[9, 17]

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Experimental Section

Amino acids were obtained from Nova Biochem (Switzerland) or Bachem (Switzerland), all other reagents were obtained from Fluka (Switzerland). Template **2** was synthesized according to published protocols.[10] Peptide **4** was synthesized according to a convergent strategy: The linear peptide Ac-$(NANP)_3$G-OH was assembled on a Sasrin resin and, after cleavage and HPLC purification, coupled to Boc-$NOCH_2CO$-Lys-OMe with PyBOP. Boc removal and HPLC purification resulted in **4** with an overall yield of 61 %. The chemical integrity was confirmed by HPLC, ESI-MS, and amino acid analysis. Compound **3** was supplied by A. Heusler (EPFL) and the monoclonal antibody Sp3E9 by Dr. G. Corradin (University of Lausanne).

SPR experiments were performed on a home-made apparatus as described previously.[18] The consecutive surface synthesis steps were performed by incubating a glass slide covered by a 40-nm thick gold film in the corresponding reaction solutions. The particular reaction was finally terminated by washing the gold surface with the corresponding solvent. Modification of the gold surface was monitored continuously on line by measuring the reflected intensity slightly off the SPR resonance angle. Surface adsorption of organic molecules shift the resonance curve to higher angles, which causes an increase in the reflected intensity (see Figure 4 as an example). In addition, angle scans were performed, which yielded the optical film thickness of the particular monolayers (details for evaluating the SPR data are given by Boncheva et al.[8c]).

FTIR spectra were recorded on a Bruker IFS 28 spectrometer equipped with a HgCdTe detector as described in detail by Liley et al.[12] One face of a Zn/Se trapezoidal ATR plate (angle of incidence 45°) was coated with a thin layer (ca 10 nm) of gold by thermal evaporation. This face was pressed against a teflon cell to form a water-tight seal. After each chemical modification of the gold surface, the ATR plate was washed with the corresponding solvent and dried under a flow of nitrogen. For each spectrum 1000 scans were recorded at 1 cm^{-1} resolution and the water vapor bands subtracted from the spectra.

TOF–SIMS spectra were recorded in triplicate on a TOF-SI mass spectrometer TRIFT,[19] designed by Ch. Evans & Comp. A liquid-metal ion source pulsed at 5 kHz was used for the production of the gallium primary ions (15kV) with a pulse width of 8 ns. The total ion dose required for one spectrum was less than 10^{11} cm^{-2}, that is, within the static regime limits of SIMS. Charge compensation was performed by injecting one low energy electron (20 eV) at every eighth ion pulse.

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