Polymer Bulletin 40, 151–157 (1998)

Polymer Bulletin


# Self-assembling functionalized templates in biosensor technology

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Received: 17 November 1997/Accepted: 12 December 1997

## Summary

A bifacial topological template exhibiting metal-binding sites and thioalkane chains has been incorporated into self-assembled monolayers and immobilised on gold surfaces: These systems allow for the detection of external ligands by SPR spectroscopy representing a first step in developing biosensors based on the TASP (Template Assembled Synthetic Proteins) concept.

## Introduction

Protein design aims to create novel proteins showing tailor-made physical, structural and functional properties (1). Often, the design process is carried out in two separate steps i.e. a stable framework ("scaffold") is constructed first and the functional properties are incorporated in a second step (2). The conceptual decoupling of structure and function is obviously hampered by the intimate relationship between primary sequence, folding mechanism and packing topology. This intrinsic dilemma explains why designers choose comparatively simple, well characterised folding motifs for probing their design strategies.

For example, the naturally occurring 4($\alpha$-helical bundle motif has become the most popular target (3) because of its versatile topology, offering a wide range of applications.

In using synthetic tools, the chemist is not restricted to the ribosomal machinery of producing linear polypeptide chains. In contrast, novel chain architectures can be created with a higher propensity for folding into a defined 3-D structure. Based on these considerations, we proposed some years ago the use of topological templates as a built-in device for directing covalently attached peptide blocks to a well-defined packing topology (4).

As a result of their branched chain architecture, these template-assembled synthetic proteins (TASP) have some unique properties(5).

Up to now, a variety of topological templates has been proposed(4,6), for example, cyclic peptides, porphyrins, cyclodextrins or more rigid polycyclic systems. The complexity of TASP molecules accessible by chemical synthesis strongly profits from recent progress in the methodology of peptide synthesis, orthogonal protection techniques and chemoselective ligation methods (7).

With the goal of extending these concepts to create TASP molecules of higher complexity exhibiting tailored functional properties, we have introduced a novel type of topological templates, termed regioselectively addressable functionalized templates (RAFT) (8). As will be shown in this article, these bifacial scaffolds allow for the independent assembly of two structural or functional modules such as a membrane channel forming helical bundle or self-assembling alkane chains and a ligand binding site. Such chimeric TASP molecules offer a

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wide range of applications in the field of peptide and protein engineering, in diagnostics as well as in biosensor- and nano-technology. Here, we focus on the development of a two domain TASP as a molecular device useful in biosensor technology.

**Results and discussion**

Recently, much interest has been focused on the construction of organised organic monolayers on metal surfaces by the process of self-assembly as elegant and versatile method for producing surfaces of well-defined composition and structure (9). In particular, self-assembled monolayers (SAM) of alkanethiols on gold form organic interfaces with properties largely controlled by the end groups of the molecules composing the film. The immobilisation of biological molecules containing SAM allow for the construction of functionalized supramolecular systems on gold exhibiting molecular recognition sites (10). Since in living organisms binding interactions occur close or at the interface of surfaces, these supramolecular systems may serve as sensing elements for the analytical detection of biomolecules. Consequently, these devices have invoked considerable interest for the characterisation of biological processes involving ligand-receptor interactions such as antibody-antigen recognition. SAM containing synthetic peptides have distinct advantages for enhancing the efficiency of binding interactions displayed by the recognition system at the monolayer interface compared to immobilised proteins (11). Since peptides of various complexity are readily prepared by chemical peptide synthesis, they can be used for surface engineering to afford tailor-made recognition properties. Here, we exploit these possibilities using a model recognition system based on immobilised metal affinity chromatography (IMAC) (12).

To this end, we functionalize gold surfaces by SAM of chelator groups and monitor the binding with an external ligand by surface plasmon resonance (SPR) spectroscopy. SPR is a powerful method for monitoring quantitatively SAM-ligand interactions detecting changes in the optical properties of a thin gold film vacuum evaporated on a glass support (13).

Applying the template concept, a self-assembling system (thioalkane) is combined with a recognition site e.g. histidine residues, with a RAFT molecule (Fig. 1). The geometry of the cycle guarantees optimal ligand accessibility of the metal ions at the monolayer interface and spatially separates the two modules (binding and self-assembling domain) (Fig. 2). The total synthesis of the two domain TASP **2** is depicted in Fig. 1.

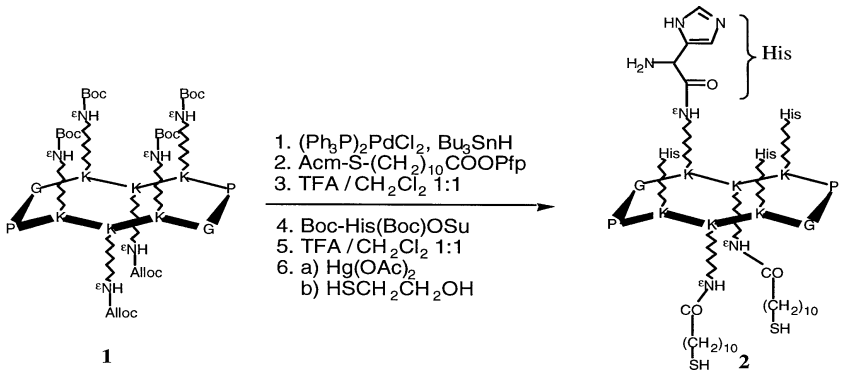


**Figure 1.** Synthesis of the chimeric TASP molecule **2** carrying histidine residues as metal binding ligands and thioalkane chains as self-assembling unity.

A combination of $N^{\varepsilon}$-Boc and Alloc-lysine side chain protection on RAFT molecule **1** was

used for selectively addressing the two faces. The Acm-protected alkane chains were introduced as pentafluorophenol active esters after Pd catalysed cleavage of the $N^{\varepsilon}$-Alloc groups. Subsequently the $N^{\alpha}$-Boc-Histidine($N^{\tau}$-Boc)-OSu was coupled; removal of the protecting groups (Boc, Acm) and HPLC purification provided the chimeric TASP molecule **2** in an overall yield of 45%.

The self-assembly process of **2** on gold surfaces was monitored in real time by SPR spectroscopy and the remaining accessible surface was subsequently blocked by immobilisation of 11-mercaptoundecan-1-ol (11, 13). Pure SAM of **2** showed a mass coverage corresponding to a mean area of 180 $Å^2$ per molecule (14). This value is in good agreement with the NMR derived template geometry (15). After loading of the histidine residues with Ni(II) ions, specific binding of a nitrilotriacetic acid (NTA) derivative to the surface is observed (Fig. 2). Desorption of this derivative upon addition of EDTA demonstrates the full reversibility of the process (Fig. 3). The change of the resonance angle Θ allowed for the estimation of the NTA stoichiometry per template. A 1:2 ratio is found consistent with results obtained in solution.

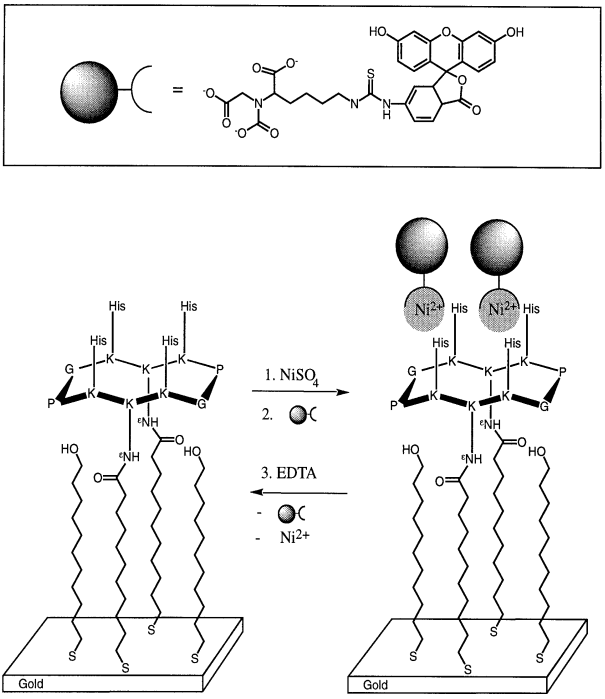


**Figure 2.** Schematic representation of a functionalized RAFT molecule forming a self-assembled monolayer (SAM) on a gold surface showing the reversible binding of NTA labelled fluoresceine ligands (box above). After loading with Ni(II), the ligand is bound to the remaining free coordination sites of the Ni (II) ions (steps 1 and 2). Removal of Ni(II) and the ligand is achieved by washing the surface with a EDTA solution (step 3). SPR spectroscopy is used to monitor the interactions of the ligand with the surface by following the distinct change of the resonance angle Θ (Figure 3).

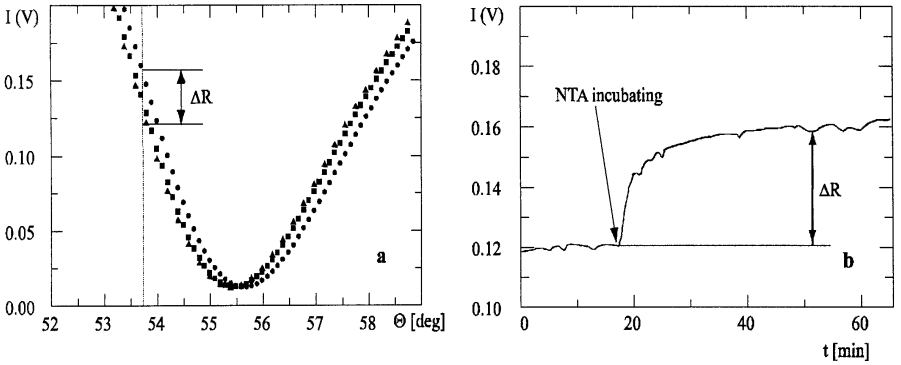


**Figure 3.** SPR measurements of surface assembled RAFT **2** (see Figure 3). (a) Reflectivity vs. angle curves for the mixed SAM of **2** and 11-mercaptoundecan-1-ol (▲), after the complexation of the NTA derivative $\Delta\Theta$=0.258 (●) and after metal removal with EDTA $\Delta\Theta$=0.068 (■). (b) Time course of the reflected light intensity during the specific binding of the NTA derivative at $\Theta$ = 53.758.

## Conclusions

The regioselective functionalization of RAFT molecules by hydrocarbon chains and metal chelating groups is studied to obtain new molecular devices for surface functionalizations. The flexibility of the synthetic methodology, based on orthogonally protected attachment sites on topological templates, allows for a wide range of modules to be assembled on the bifacial template molecule. Self-assembly of the chimeric TASP molecule **2** (Fig. 2) on gold surfaces provides a supramolecular array of oriented metal-binding sites. This surface recognises and reversibly binds external ligands as monitored by SPR. Consequently, molecular systems with tailored physical and chemical functions can be obtained according to the elaborated concept offering interesting perspectives in the area of biosensor- and nano-technologies.

**Acknowledgements**: This work was supported by the Priority Program Biotechnology of the Swiss National Science Foundation.

## Experimental

*Surface plasmon resonance (SPR).* The surface plasmon resonance measurements were performed, using the Kretschmann coupling scheme (16) on a home-made computerised reflection apparatus as described in details elsewhere(17). At the resonance angle the incident laser beam (He-Ne laser, 632.8 nm, p-polarised) couples via an equilateral, high index prism (SF 10, n=1.723) to the surface plasmon mode in a thin gold film (40 nm).

*Layer formation.* For the self-assembly of peptide and 11-mercaptoundecanol, a gold film (40 nm), vacuum evaporated on a cleaned glass slide, was incubated in peptide or thiol water/methanol mixtures. After 1h self-assembly, the surface was extensively washed with water / methanol. Finally a solution of thioalcohols in methanol / water was added to block remaining defects in the monolayer. The binding was monitored *in situ* using surface plasmon resonance. For $Ni^{2+}$ binding the surface was incubated with 100 MM $NiSO_4$ solution for 10 min. After washing, the cell was calibrated with buffer and the fluoresceine-labelled NTA was added. The surface was washed with buffer and the final thickness measured. The His-exposing surface was regenerated by washing with 0.5 M EDTA in buffer.

***cyclo*[K(Boc)-P-G-K(Boc)-K(Aloc)]$_2$** *(T-(Boc$_4$Aloc)$_2$)* **(1)**

Using Fmoc-protected amino acids, the linear decapeptide was synthesised by solid-phase peptide synthesis on 2.5g Sasrin® Resin The first amino-acid Nα-Fmoc-Lys(Aloc)-OH (2 equiv.) was coupled to the anchor using PyBrop (2.1 equiv.) in the presence of 4-Dimethyl-aminopyridine (0-3 equiv.) and diisopropylethylamine(DIEA) (5 equiv.) in extensively degassed N,N-Dimethylformamide (DMF). The peptide chains were then assembled by sequential couplings of Nα-Fmoc-amino acid (2 equiv.) for 45 to 60 min at room temperature with PyBop (2.1 equiv). The completeness of each coupling was verified by the Kaiser test (18). The Nα-Fmoc protecting groups were removed by treatment with piperidine (20% v/v in DMF, 3 min then 15 min cycle); the completeness of each deprotection being verified by the UV absorption of the piperidine washing at 302 mm (19). The protected peptide was then cleaved from the resin with 1% TFA in DCM (15 ml, 6 cycles of 10 min) and was neutralised with 1% pyridine in DCM (6x15 ml). The solvent was removed under reduced pressure and 2.50g (69%) of the desired peptide was obtained by precipitation from DCM-diethyl ether. $t_R$ = 19.4 min (gradient 25% B to 100% B in 30 min), ESI-MS: 1664.2 (M+H), 832.8 ((M+2H)/2). A solution of the linear peptide (1.0 g, 0.60 mmol), PyBop (350 mg, 0.67 mmol) in DMF (1000 ml) was treated at room temperature with a solution of DIEA (400 µl, 2.4 mmol). After 90 min the cyclization was complete as determined by analytical HPLC, and the solution was concentrated under reduced pressure. the residue was dissolved in DCM and washed with NaOH (1N), HCl (1N) and $NaCl_{sat}$, precipitation with diethyl ether, and chromatography column purification ($CHCl_3$ / MeOH, 9 / 1) afforded 780 mg, (76%) of the cyclic decapeptide (**1**). $t_R$ = 24.6 min (gradient 25% B to 100%B in 30 min), Rf = 0.35 ($CHCl_3$ / MeOH = 9 / 1), MS: 823.8 (M/2+1), m.p. > 250°C, ASA: G 1.74 (2), K 6.0 (6), P 1.83 (2).

**T(His)$_4$(CO-(CH$_2$)$_{10}$-SH)$_2$ (2)**

*Acm-S-11-mercaptoundecanoic acid.:*

11-mercapto-undecanoic acid (1.00 g, 4.58 mmol) and acetamidomethanol (409 mg, 4.58 mmol) were dissolved in 50 ml trifluoroacetic acid and stirred for 30 min at room temperature. After solvent removal the residue was dissolved in $CHCl_3$ and extracted with 2N NaOH. The aqueous phases were acidified and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$ and the solvent removed under pressure. The product was crystallised with DCM / petrol ether to afford 950 mg (72%) of a white powder. Rf = 0.26 (diethyl ether / acetic acid = 98 : 2), m.p. 81°C, $t_R$ = 17.19 (gradient 25% B to 100% B in 30 min), ESI-MS: 290.5 (M+1), 312.4 (M+Na), $^1$H-NMR: 1.15 (m, 12H, H alkane chain), 1.27 (m, 6H, H alkane chain), 1.95 (s, 3H, $CH_3CO$), 2.30 (td, 2H, S-CH2- alkane chain), 4.30 (d, 2H, N-$CH_2$-S), 4.6 (s, large, 1H, acid), 5.75(s, large, 1H, NH).

*Acm-S-11-mercaptoundecanoic-pentafluorophenyl ester:*

Acm-S-11-mercapto-undecanoic acid (100 mg, 346 mmol) pentafluorophenol (70 mg, 380 mmol) and DCC (206 mg, 380 mmol) were reacted in $CHCl_3$ (4 ml). The solution was filtered after 12 h, extracted with a solution of 5% $KHSO_4$, 10% $NAHCO_3$ and $NaCl_{sat}$ and the organic phase dried over $MgSO_4$. After removal of the solvent 99 mg (63%) of the desired active ester was obtained. Rf = 0.7 (diethyl ether / acetic acid = 98 : 2), m.p- 67-68°C, $t_R$ = 17.59 (gradient 25% B to 100% B in 30 min), ESI-MS: 456.7 (M+1.), $^1$H-NMR: 1.15 (m, 12H, H alkane chain), 1.22 (m, 6H, H alkane chain), 1.95 (s, 3H, $CH_3CO$), 2.30 (td, 2H, S-$CH_2$-alkane chain), 4.30 (d, 2H, N-$CH_2$-S), 5.75(s, large, 1H, NH).

*T(His)$_4$(CO-(CH$_2$)$_{10}$-S-ACM)$_2$*:

The Aloc groups were removed according typical procedure (20) ($PdCl_2(PPh_3)_2$ / $BU_3SnH$). A solution of the deprotected template (40 mg, 27 µmol), Acm-S-11-mercaptoundecanoic-acid pentafluorophenol ester (25 mg, 55 µmol), DMAP in dry DMF was treated with DIEA (100

µl, 580 µmol). The solvent was removed after 9 h and the residue precipitated with diethyl ether to afford 50 mg (91%) of the product. $t_R$ = 24.82 min (gradient 25% B to 100% B in 30 min), ESI-MS: 1010.6 ((M+2H)/2). The peptide (50mg, 25 mmol) was then dissolved in 10 ml of 50% TFA solution in DCM for 45 min. After removal of the solvent under reduced pressure, the residue was precipitated with diethyl ether and lyophilised to yield 41.8 mg (81%) of Boc deprotected peptide. $t_R$ = 13.99 min, ESI-MS: 810.7 ((M+2H)/2). Nα-Boc-His(Boc)-OSu was coupled with DIEA to the free-amino peptide at room temperature. The reaction was complete after 3 h and after removal of the volatile the residue was treated with a 50% solution of TFA in DCM during 45 min. The solvent was evaporated and the remaining residue was purified by preparative RP-HPLC to yield 12 mg (57%) of pure $T(His)_4(CO-(CH_2)_{10}$-S-Acm$)_2$.peptide. Acm protecting groups were removed with $Hg(OAc)_2$ (20 mg, 50 µmol) d for 2h in 5 ml 30% acetic acid under $N_2$ atmosphere. Then, β-mercaptoethanol (430 µl, 4.8 mmol) was added and the solution stirred overnight. The solution was filtered and purified by preparative RP-HPLC to afford 12 mg (64%) of **2**. $t_R$ = 16.65 min (gradient 0% B to 100% B in 40 min), ESI-MS: 507.0 (M/4+1), 676.6 ((M+3H)/3), 1014.2 ((M+2H)/2).

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