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Self-assembling monolayers of helical oligopeptides on gold with applications in molecular electronics†

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A generic strategy is outlined for preparing a chiral functionalised surface using self-assembled monolayers (SAMs) of helical oligopeptides for intended application in molecular electronics, sensors and catalysis. Helical peptides have been designed and synthesised with 3 Met or 2 Cys residues per peptide, placed in the sequence so that in a helical conformation their side-chains are on the same side of the helix and organised to bind to gold by self-assembly of the thioether or thiol functions. Circular dichroism (CD) of the Met-containing peptides showed that they are mainly helical in organic solvents and FT-RAIRS (reflection–absorption infrared spectroscopy) of their SAMs on gold confirmed that they are aligned with the helix axes parallel to the surface. However complete coverage of the surface is dependent critically on the preparation of the gold. CD of an oligopeptide containing 2 Cys residues suggested that the conformation was a mixture of helix and random coil. Films of this peptide adsorbed from different solvents were characterised by ellipsometry and cyclic voltammetry. Ethanol and methanol led to multilayer formation indicating protic solvents destabilise the helical structure, allowing formation of intermolecular disulfide bonds. By contrast the aprotic solvent acetonitrile led to formation of a close-packed self-assembled monolayer suitable for elaboration into a functionalised supramolecular architecture.

As the size of electronic components decreases, approaches have been made to design and prepare molecules that possess properties that are useful in electronic circuits.[1] These efforts may be split broadly into two areas,[2] molecular materials for electronics, which use the macroscopic properties of the materials, and electronics at the molecular level which exploits the microscopic properties of individual molecules.[3] With respect to the second area, various ingenious ideas have been proposed for using molecules as a rectifier[4] or a shift register[5] and some molecules have been demonstrated to act as switches[6–13] and storage devices.[14,15] However most of these have been in solution. In a functioning computer it is essential that the memory, input, output and processing units are at well-defined positions to enable them to communicate with each other. This indicates a solid state device. In this paper we present a plan to use self-assembled monolayers (SAMs) of helical oligopeptides to produce a surface on which various functional groups may be positioned with nanoscale resolution. We envisage that this may also be of utility in fields such as sensors, biomaterials and catalysis.

Our strategy is to prepare helical oligopeptides that contain both attachment sites for molecular computing elements and sulfur containing side-chains to promote self-assembly on gold. By using solid-phase peptide synthesis these different groups can be introduced into the sequence at predefined sites relative to each other. In an ideal α-helix one turn around the helix axis encompasses 3.6 residues. Thus, placement of three S-containing residues at positions i, $i+4$ and $i+7$ will ensure their side-chains all extend from the same side of the helix, preorganised to bind to a gold surface. Including the residue modified with the molecular computing element at $i+2$ will result in this group lying on the opposite side of the helix so that following self-assembly it will be at the ambient/film interface. To achieve lateral positioning of different components it will be necessary to specifically link the peptides in solution prior to the self-assembly step. Methods are available to link the peptides through either covalent or non-covalent interactions. Possibilities include forming a peptide bond between the side-chains bearing amine and acid groups (*e.g.* Lys and Glu) or using amino acids bearing complementary hydrogen bonding arrays such as those found in nucleic acids. The supramolecular structure will ultimately be confined to a surface by sulfur-mediated self-assembly on gold. The overall strategy is summarised in Fig. 1.

Our approach relies on the fundamentals of self-organisation prevalent in Nature, which uses information 'encoded' in the structures of simple molecules to direct the formation of much larger architectures with known conformation, *e.g.* proteins, nucleic acids or cells. Thus choice of peptide sequence 'self-organises' the molecules into a helix; specific linking of helices in solution may be performed using complementary molecular recognition, and the final step is self-assembly of the supramolecular architecture onto a surface using the specificity of the sulfur–gold interaction.

Self-assembling monolayers of sulfur-containing molecules on gold are attractive systems for tailoring surface properties and the study of interfacial phenomena, *e.g.* wetting and adhesion,[16–19] and fundamentals of electron transfer.[20–22] Most efforts have concentrated on the formation, structure and properties of monolayers of long-chain alkanethiols.[23,24] These systems have the advantages[16] that a strong, specific interaction between the adsorbate and the substrate drives spontaneous formation of a monolayer, the films are densely packed and physically robust and a variety of other functional groups may be accommodated within the monolayer. Examples of other functional groups include alkanethiols with terminal ferrocenylazobenzenes,[25] tetrathiafulvalene,[26] viologens,[27] biotin[28] and thiol-terminated oligoimides[29] and oligo(phenyleneethynylene)s.[30] The potential of exploiting these structures in sensors[31–34] and biomaterials[18] has been recognised.

Terminally-modified alkanethiols and other molecules where the long molecular axis extends out from the metal surface still present a homogeneous surface to the organic/ambient interface. Although the mole fraction composition of mixed

†Peptide purification and characterisation details are available as supplementary data (SUPPL. NO. 57510, pp. 9) from the British Library. For details of the Supplementary Publications Scheme, see 'Information for Authors', *J. Mater. Chem.*, available *via* the RSC web page (http://www.rsc.org/authors). For direct electronic access see http://www.rsc.org/suppdata/jm/1999/1097/.

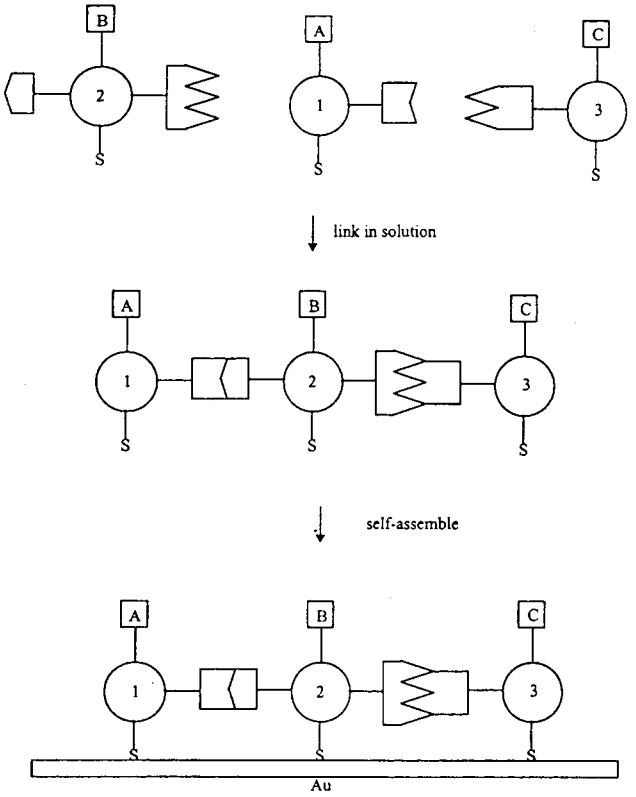


Fig. 1 Proposed preparation of an ordered, functionalised surface with nanometre scale features using helical oligopeptides. The schematic is viewed along the helix axes of peptides **1**, **2** and **3** which have side-chains modified to carry functional elements A, B and C respectively on the opposite sides of the helices to thiol or thioether groups. The peptides are specifically linked in solution through complementary binding groups to form discrete supramolecular assemblies. These are then locked together and immobilised *via* formation of a self-assembled monolayer on gold using the thiols or thioethers. The resultant surface confined molecular architecture will contain functional elements A, B and C placed at known relative positions a few nm apart. This plan could be extended to include more functional elements by increasing the number of different peptides or by including several elements on each peptide chain.

monolayers of alkanethiols co-adsorbed with terminally functionalised thiols may be controlled empirically, the relative positioning of the two components is still random.[35]

In the few previous examples of SAMs of peptides that have recently been reported, the peptides generally had only one attachment point to the gold surface, either through the side-chain of a terminal Cys residue[34,36–38] or *via* a thioalkyl carboxylic acid coupled to the N-terminal.[33,39–41] Outside our own work, one notable exception was the recent report from the Mutter and Vogel laboratories,[42] which described the formation and characterisation of a SAM of a cyclic peptide template with 2 or 4 pendant alkylthiolates covalently bound to side-chains of the template. The solution side of the template was derivatised with metal-binding residues to which the binding of metals and subsequent ligands was demonstrated. Although it was not discussed in their paper this system could also be extended to produce a functionalised surface similar to that we have proposed.

As our primary objective we have concentrated on investigating the formation and structure of SAMs of monomeric helical oligopeptides aligned with the helix axis parallel to the surface. We have previously described the formation and characterisation of SAMs of the helical oligopeptide Fcb-13Met3 (**1**), Fcb-Ala-Aib-Ala-Met-Aib-Ala-Ala-Met-Ala-Aib-Met-Ala-Ala-NH_2 (where Fcb is ferrocenylbutyric acid).[43] This 13 residue peptide contained three Met residues (hence 13Met3) positioned so their side-chains aligned along one side of the helix and formed stable monolayers on gold with the helix axis parallel to the surface as characterised by electrochemistry and reflection–absorption infrared spectroscopy (RAIRS). In this full paper we describe the design and preparation of SAMs of other helical oligopeptides containing several Met or Cys residues and their characterisation by cyclic voltammetry (CV), RAIRS and ellipsometry. The conditions required for monolayer formation of each type of peptide through the side-chain sulfur functions are discussed.

Results and discussion

General design principles

Helical peptides are attractive building blocks with which to construct a functionalised surface as their synthesis and structure have been well studied. The more common α-helix is stabilised by hydrogen bonds between the main chain N–H of the $(i+4)$th and the main chain C=O of the ith amino acid in the sequence and one turn around the helix axis encompasses 3.6 residues. An alternative known helical geometry is the 3_{10}-helix[44,45] so called because 3 residues form one complete turn around the helix axis and there are 10 atoms within the intramolecular ring formed by the hydrogen bond between N–H of residue $i+3$ and the C=O of residue i. The differences in pitch and hydrogen bonding mean that a 3_{10}-helix is longer and thinner than an α-helix of the same number of residues. The conformation of a peptide is determined by its amino acid sequence. In particular, peptides consisting entirely of amino acids with hydrocarbon side-chains are known to adopt helical structures in organic solvents.[44,45] The 20 α-amino acids present in mammalian proteins offer a variety of side-chain functions, some of which (*e.g.* Lys, which contains the NH_2 group, and the CO_2H of Glu) may be readily modified to introduce other desired functions. Two amino acids, Cys and Met contain sulfur functions in their side-chains (-CH_2SH and -$CH_2CH_2SCH_3$ respectively) which are expected to form strong bonds with a gold surface *via* self-assembly. Of these two Met was initially the more attractive. The greater separation between the peptide backbone and the sulfur atom in Met compared with Cys should allow the sulfurs to find optimal positions on the gold surface. It should also reduce unfavourable steric interactions between the metal surface and other side-chains on that side of the helix. Furthermore, although the thiol of Cys binds more strongly to gold than the thioether of Met, it was anticipated that the biomimetic route of multiple weak interactions might be a more efficient route to well ordered systems. In this paper we compare the formation and structure of SAMs of peptides containing both types of residue.

Met-containing peptides

Our prototype sequence was that of peptide **1**, Fcb-13Met3. Our aim of adsorbing the peptides to gold in a helical conformation *via* the thioethers placed certain restrictions on the primary structure. In particular, alignment of the surface-binding side-chains along the same side of the α-helix such that they could bind cooperatively to a gold surface (Fig. 2) required Met to be included at positions 4, 8 and 11. The designed formation of three sulfur–gold bonds was expected to introduce an element of selectivity as helical conformations should be bound more strongly than other conformations where only one or two peptide–surface interactions are possible. The remaining amino acids used, Ala and Aib (α-aminoisobutyric acid) are known strong helix formers in organic solvents[44,45] and have small methyl side-chains to minimise unfavourable steric interactions with the surface. The N-terminus was acylated with ferrocenylbutyric acid to provide a convenient electrochemical label. Peptide **2**, Fcp-12Leu2, Fcp-Ala-Aib-Ala-Ala-Leu-Aib-Ala-Ala-Ala-Aib-Leu-Ala-

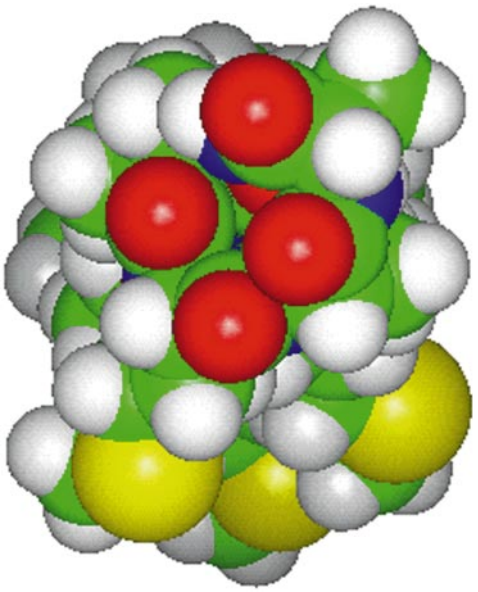


Fig. 2 Space-filling model of **1**, 13Met3 in an α-helical conformation showing the alignment of the 3 S atoms along the underside of the helix. The main axis of the helix is normal to the plane of the page. The N-terminal group here, acetyl, is shown instead of the actual one (4-ferrocenylbutyric acid) for clarity. The atom colours are C: green, H: white, N: blue, O: red, S: yellow.

NH_2 (where Fcp is ferrocenylpropionic acid), available from previous studies (J. Reid, D. Nonhebel and B. D. Moore, unpublished results) provided a convenient, non-sulfur-containing control of similar length, composition and secondary structure[43] to **1**.

As described previously[43] both peptides **1** and **2** were synthesised efficiently by conventional solid phase methods. The circular dichroic spectra of Fcb-13Met3 confirmed that it was mainly helical in both methanol and acetonitrile. SAMs of **1** were prepared on a gold disk electrode and were found to be remarkably stable to repetitive cycling in ethanol–electrolyte. In contrast the initial surface coverage of peptide **2** was typically less than 15% of peptide **1** and rapidly decreased during cycling under the same conditions. In the RAIRS spectrum of a SAM of the Met-containing peptide on evaporated gold the amide I band was at 1664 cm^{-1}, indicative of a 3_{10}-helix, and the amide II and amide III bands were at 1545 cm^{-1} and 1264 cm^{-1} respectively. Comparison of the reflectance with the transmission spectrum measured of **1** dispersed in KBr confirmed that the helix axis was parallel to the surface, the orientation expected for a self-assembled layer. In contrast the RAIRS of Fcp-12Leu2 suggested no particular orientation of the peptide at the surface.

Following these promising results we sought to design completely α-helical Met-containing peptides and to prepare SAMs with greater coverage of the surface. To increase the α-helicity the length of peptide **3**, 16Met3, Ala-Ala-Aib-Ala-Met-Ala-Phe-Ala-Met-Aib-Phe-Met-Aib-Ala-Ala-Ala-NH_2, was increased to 16 residues as longer peptides favour the α- over the 3_{10}-helical conformation.[44] Two Phe residues (side-chain CH_2Ph) were placed at positions 7 and 11 in the sequence where they could potentially stabilise the α-helical form by solvophobic packing of the side-chain in polar solvents such as acetonitrile and methanol. Although in proteins it is more usually found in β-sheets Phe has been used in organic-soluble helical peptides.[44,46] The sequence of peptide **4**, 16Nle3, Ala-Ala-Aib-Ala-Nle-Ala-Phe-Ala-Nle-Aib-Phe-Nle-Aib-Ala-Ala-Ala-NH_2, differed from that of **3** only by replacement of the three Met residues with the isosteric Nle (side-chain $-CH_2CH_2CH_2CH_3$) and was chosen to provide a close comparison of the adsorption of peptide **3** without the gold-binding thioether groups.

Assembly of each sequence on the solid phase proceeded smoothly and small portions of each Fmoc-peptide-resin (Fmoc = fluorenylmethoxycarbonyl) were deprotected and acylated as needed to provide the *N*-acetyl (Ac), *N*-(4-ferrocenylbutanoyl) (Fcb) or *N*-*p*-nitrobenzoyl (NB) derivatives. As with peptides **1** and **2** cleavage of the ferrocene derivatives was carried out under oxygen-free conditions to minimise the oxidation of the ferrocene to ferrocenium. The Met-containing peptide contained small quantities of the sulfoxide. After purification by reversed-phase HPLC, derivatives of both peptides were only sparingly soluble in a range of solvents. Amphipathic helical peptides are known to aggregate in water to form bundles with the hydrophobic surfaces shielding each other from the solvent.[47–49] These completely non-polar sequences may have aggregated in a similar manner even in these less polar solvents (methanol and ethanol; acetonitrile and ethyl acetate were also tried but no dissolution could be detected). Formation of β-sheets, which are often believed to aggregate and cause precipitation, was considered unlikely in peptides containing several Aib residues.[44]

The circular dichroic (CD) spectra were measured using the NB derivatives, since here the peptide concentration could be determined accurately from the UV-absorbance of the NB group. Each spectrum (Fig. 3) showed the double-minima at 208 and 222 nm typical of α-helices.[50] The value of $[\theta]_{222}$ of −23 300 for NB-16Met3 demonstrated the expected increase in helicity over Fcb-13Met3 (for which $[\theta]_{222}$ was −18 300).

Films of Ac-16Met3, Ac-**3**, and Ac-16Nle3, Ac-**4**, were prepared on evaporated gold slides by immersion in saturated peptide solutions for 2 days. 'Cast' films of each peptide were also prepared by pipetting their solutions onto slides and allowing the solvent to evaporate. The reflectance spectra of the cast films were taken as being representative of disordered films (Fig. 4). The amide I bands at 1663 cm^{-1} and 1667 cm^{-1} for Ac-**3** and Ac-**4** respectively are in the region more typical of 3_{10}-helices[51] but may also indicate α-helices. The latter are characterised by an amide I maximum at 1650–1658 cm^{-1}, but this may be shifted to higher frequency by *ca.* 10 cm^{-1} after adsorption on gold.[38] As expected no bands were detected in the reflectance spectrum of the slide immersed in Ac-**4**. Gratifyingly in the surface spectrum of Ac-**3** an amide I band

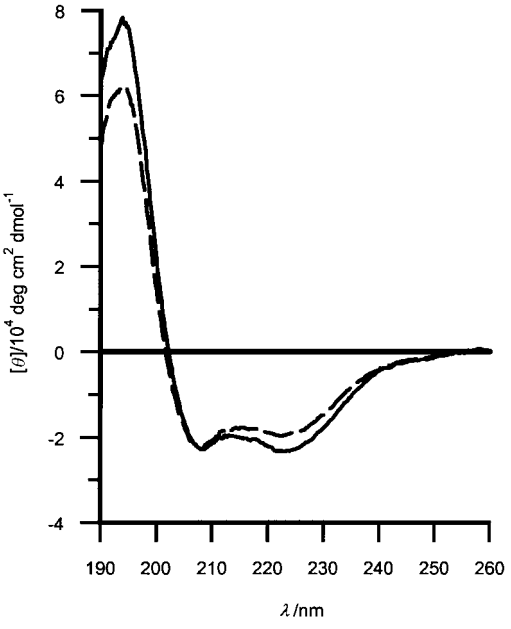


Fig. 3 Circular dichroic (CD) spectra of peptides NB-**3** (continuous line, 130 μM) and NB-**4** (dashed line, 180 μM) in methanol at room temperature.

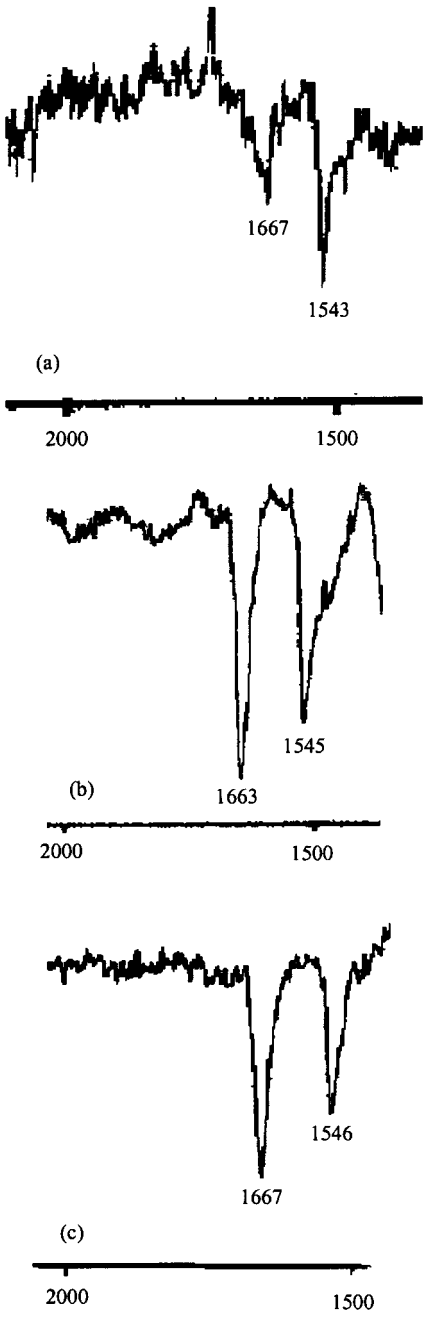
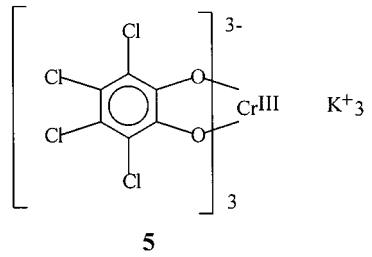


Fig. 4 FT-RAIRS (displayed as % transmission spectra) of (a) Ac-16Met3 self-assembled from a 1 mM solution in methanol; (b) cast film of Ac-16Met3; (c) cast film of Ac-16Nle3. The *x*-axes units are cm^{-1}.

appeared at 1667 cm^{-1}. Furthermore its intensity was significantly less than that of the amide II band.

The RAIRS technique allows the orientation of the peptides to be determined as only vibrations with a component normal to the surface are enhanced.[52] The main component of the amide I band is stretching of the carbonyl bonds and in a helical peptide these will be aligned along the helix axis. The main contributions to the amide II band are in-plane bending of N–H and C=O, and C–C and C–N stretching.[53] For each of these vibrations the transition dipole moment subtends a range of angles to the helical axis. In the self-assembled peptide monolayers binding *via* the methionine side-chains is expected to align the helical axis parallel to the surface. This is expected to lead to a dramatic reduction in the intensity of the RAIRS amide I band relative to the amide II compared to the spectrum of the cast film. It can be seen in Fig. 4 that this is exactly what is found for the SAM of Ac-**3** and supports the assignment of the amide I band at 1667 cm^{-1} to a helical conformation. Similar changes were observed by Boncheva and Vogel who prepared monolayers of helical peptides by the Langmuir–Blodgett and self-assembly techniques, employing a Cys at either the N- or C-terminus.[38] Their more detailed theoretical analysis reached the same conclusions as ours and was supported by surface plasmon resonance measurements.

The surface coverage and stability of the adsorbed peptide films were assessed by cyclic voltammetry of the ferrocene derivatives. In addition we investigated the ability of a SAM of Ac-16Met3 to block electron transport to a couple in solution. The working electrode used in this experiment was an evaporated gold slide which had been immersed in peptide solution (0.04 mM in methanol for 17 h) and rinsed. Voltammetry of the modified slide in a solution of $Ru(NH_3)_6Cl_2$ showed no significant difference to that recorded at bare gold. Although electron transfer to this complex has previously been shown to be blocked by alkanethiol SAMs these present a much denser, thicker barrier than a close-packed monolayer of helical peptide ($C_{21}H_{43}SH$ forms a monolayer approx. 30 Å thick compared to the peptide diameter of 12 Å).[54] The voltammograms of the much larger couple **5** $[Cr(Cl_4C_6O_2)_3]^{3-}$ (chromium tris(tetrachlorocatecholate)$_3^{3-}$) at a bare gold slide and at the peptide-modified electrode are shown in Fig. 5. Clearly the peptide monolayer strongly hindered electron transfer to and from this bulky Cr complex indicating that a coherent monolayer had adsorbed over the whole electrode. Following this encouraging observation we investigated the binding of **3**, Fcb-16Met3, to a gold disk electrode. However despite intensive efforts using a wide variety of surface preparations, solvents and immersion times the measured surface coverages obtained were no better than that of peptide **1**.



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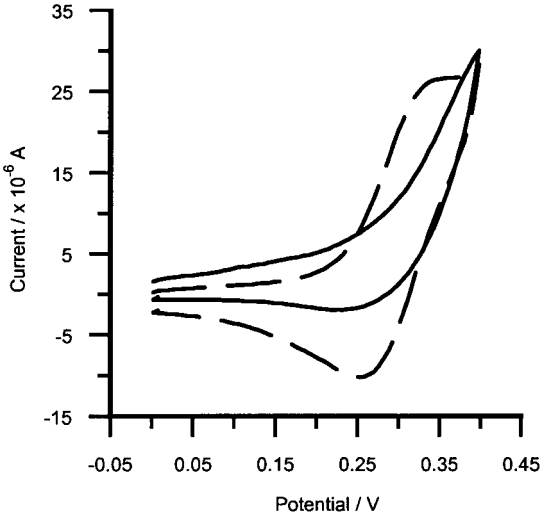

Fig. 5 Cyclic voltammograms (CVs) of $Cr(Cl_4C_6O_2)_3^{3-}$ at (dashed line) a bare gold electrode and (continuous line) gold covered with a SAM of Ac-16Met3. CVs were recorded at 50 mV s^{-1} in saturated KCl in 9 : 1 methanol–water. The reference electrode was Ag/AgCl.

Other groups have previously found that thioether monolayers failed to completely displace contaminants from the gold surface[55] and that the preparation on gold of SAMs of resorcin[4]arenes through 4 pendant dialkyl thioether arms has been reported to be of low reproducibility due to the critical conditions required for the electrode preparation and for self-assembly.[31] In contrast the more frequently used thiolates have been reported to bind strongly to gold and are able to displace contaminants from the surface.[55] In model experiments with Fcb-derivatives of Met and cystine esters we too observed significantly stronger binding of the thiolate over the thioether.[56] We thus decided to investigate the possibility of generating SAMs from an oligopeptide containing two Cys residues as surface binding groups, the expectation being that we might more readily prepare films with high stability and surface coverage than those prepared from peptides **1** and **3**.

Cys-containing peptide

The sequence of oligopeptide **6**, 16Cys2, Ala-Ala-Aib-Ala-Phe-Ala-Cys-Phe-Leu-Cys-Aib-(NO_2)Phe-Ala-Aib-Leu-Ala-OH, where (NO_2)Phe is *p*-nitrophenylalanine, was based on a peptide, PRM1 (Ac-Ala-Ala-Aib-Ala-Phe-Ala-Acc-Leu-Aib-Ala-Don-Ala-Aib-Leu-Ala-NH_2, where Don and Acc are Glu residues modified with electron donating and accepting groups, respectively) which was characterised as forming a 3_{10}-helix in acetonitrile.[10] In contrast to peptides **1** and **3**, with peptide **6** molecular modelling (Fig. 6) indicated that binding of the two Cys side-chains to gold would be more favourable in a 3_{10}-helix rather than an α-helix. The two Cys residues were thus placed at positions *i*, *i*+3 where we envisaged that formation of an intramolecular disulfide bond between them could be used to both stabilise the 3_{10}-helical conformation and protect the thiol function (*e.g.* from intermolecular disulfide formation) until contact with gold cleaved the S–S bond. The end sequences of PRM1 were conserved and two modified Glu residues with large electron donor and acceptor substituents were replaced with Phe and (NO_2)Phe. These were chosen as their side-chains are relatively bulky (which might disrupt the hydrophobic aggregation responsible for the low solubility of 16Met3) but did not require protecting groups, thus keeping the synthesis as simple as possible. The *p*-nitrophenylalanine residue served the dual purpose of enabling us to measure accurately the concentrations of the peptide solutions due to its strong UV absorption and to act as an infrared probe of the peptide's orientation on the surface. The four other residues on the side of the helix intended to bind to gold were Ala, which should offer the least unfavourable steric interactions with the surface.

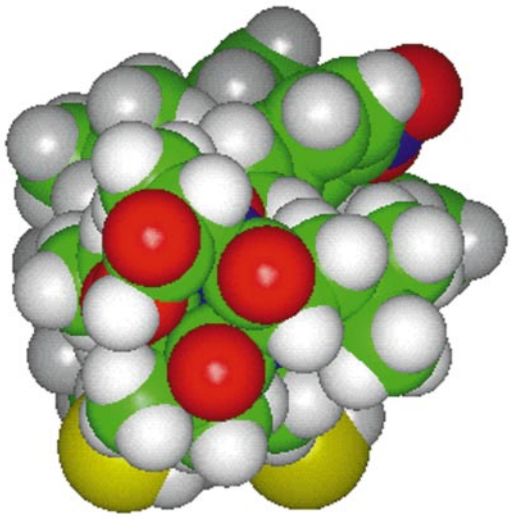

Fig. 6 Space-filling model of **6**, Ac-16Cys2 in a 3_{10}-helical conformation showing the alignment of the 2 S atoms on the underside of the helix. The main axis of the helix is normal to the plane of the page. The atom colours are C: green, H: white, N: blue, O: red, S: yellow.

Using Cys complicates the synthesis as the strongly nucleophilic thiol must be protected. Other potential difficulties may occur during the subsequent removal of the protecting group and manipulation of the peptide-dithiol, which may oxidise to the disulfide. The shorter spacer group between the peptide backbone and the sulfur (one methylene group rather than two in Met) may also introduce difficulties during the self-assembly step. Unfavourable steric interactions between the peptide and surface could prevent the two sulfur atoms from simultaneously accessing ideal binding positions on the gold surface.

The peptide was prepared on the solid phase. Both cysteine residues were protected with the trityl group (Trt, triphenylmethyl)[57] which may be cleaved either by TFA (trifluoroacetic acid) to afford the thiol or by iodine to form the disulfide. These methods avoid the use of toxic reagents (Hg^{II}, Tl^{III}) or thiols; we were particularly keen to avoid the latter as even small quantities could compete with the peptide for binding to gold. The peptide-resin was stored Fmoc- and ditrityl-protected (Fmoc-16Cys2{Trt}$_2$-OR) and subsequent modifications were carried out as required. From herein we will refer to the peptide dithiol as 16Cys2($SH)_2$ and the peptide disulfide as 16Cys2(S_2).

Cleavage was attempted first with 95% aq. TFA without protection from the atmosphere with triisopropylsilane (TIPS) preferred to ethanedithiol as the scavenger.[58] The peptide-dithiol was obtained as a yellow and green solid indicating that some oxidation of the ferrocene had occurred. ES-MS of the crude revealed that as well as the desired product peptides lacking Aib and Fcb were also present in minor quantities. Attempts to purify the mixture by HPLC in methanol–water and by crystallisation were unsuccessful and a sample of the crude material was adsorbed to the disk electrode and analysed by cyclic voltammetry, as described below.

The cyclisation by disulfide formation of short β-turn-forming peptides has been carried out on the resin using iodine by Albericio and co-workers, the 'pseudo-dilution' conditions favouring the intramolecular process.[59] The similarities of these researchers' sequence to ours (containing 2 Cys at *i*, *i*+3) and the excellent yields of disulfide-peptide that they obtained after acidolytic cleavage made this an attractive starting point. Thus Fmoc-16Cys2($Trt)_2$-O-resin was treated with a solution of iodine (2 equiv. per Cys) in acetonitrile at 0 °C and subsequently deprotected and acylated with Fcb-OH. After cleavage with 95 : 5 TFA–water analysis of the crude material by Electrospray Ionisation Mass Spectrometry (ESI-MS) confirmed that the desired peptide had been obtained. It is notable that effecting the disulfide formation, deprotection and acylation steps in dimethylformamide (DMF) rather than acetonitrile produced undesired intermolecular disulfide bonds. This was established by the presence of 3+ and 4+ ions of the dimeric peptide in the ESI-MS. Other insoluble material may have been higher oligomers. In retrospect this is not too surprising as DMF hydrogen bonds to main chain N–H groups, destabilising the helix and allowing the peptide to access more extended conformations in which the S–S bonds may form between different molecules. Alternatively as a weakly basic solvent DMF may have 'scrambled' the initially formed intramolecular bonds.

The CD spectra of **6**, Ac-16Cys2(S_2) in methanol, ethanol and acetonitrile (Fig. 7) indicated that the peptide conformation was a mixture of helix and random coil. The intensities of the minima at 222 nm (−11 000 to −12 000) showed that the helicity was significantly less than the Met-containing peptides. However, the unstructured sections of mixed helical–random coil peptides tend to be near the termini,[60] and it was anticipated that since in peptide **6** the dicysteine gold-

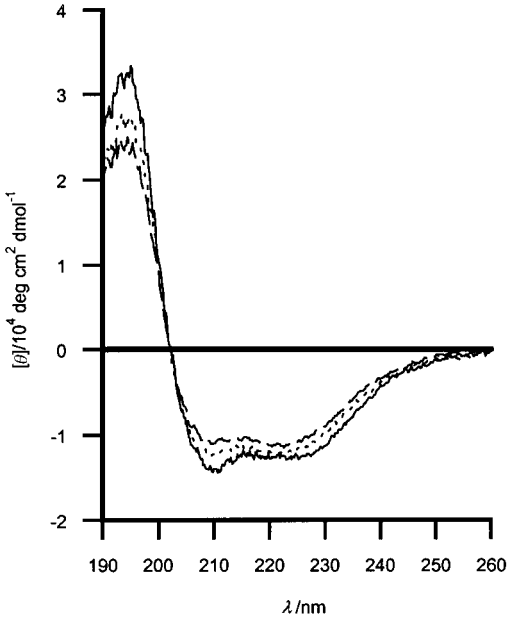

Fig. 7 Circular dichroic spectra of peptide **6**, Ac-16Cys2(S_2) in methanol (long dashes, 190 μM), ethanol (short dashes, 160 μM) and acetonitrile (continuous line, 160 μM) at room temperature.

binding site lies in the middle it may be unperturbed. Despite our aim of designing a 3_{10}-helix the ratio of the intensities of the minima at 208 nm and 222 nm of *ca.* 1 is more typical of an α-helix; in 3_{10}-helices it is often 0.5 to 0.8.[61,62] It is probable that at 16 amino acids, 16Cys2 is sufficiently long that the α-helix is the favoured conformation.

A self-assembled film of Fcb-16Cys2(SH)$_2$ on a gold disk electrode was prepared using a saturated solution of freshly cleaved peptide in methanol. CV of the unrinsed electrode after 2 days did not initially contain any faradaic waves but after repeated cycling the oxidation and reduction waves of the ferrocene became discernible (Fig. 8). After 10 days storage in a sealed jar the CV was measured in organic solution (*n*-Bu_4NBF_4 in acetonitrile, Fig. 8). As before several potential sweeps were required to condition the film and reveal the ferrocene peaks, which were still unexpectedly broad. From

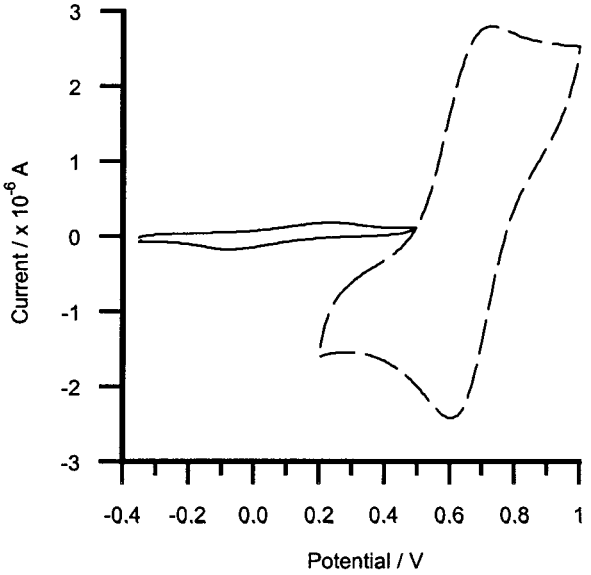

Fig. 8 Cyclic voltammograms of Fcb-16Cys2(SH)$_2$ film on a gold disk electrode *vs.* Ag wire reference electrode. Continuous line: 20 min after rinsing, measured at 50 mV s^{-1} in 0.1 M aq. $NaClO_4$. Dashed line: 10 days after rinsing, 10th scan at 500 mV s^{-1} in 0.1 M tetra-*n*-butylammonium tetrafluoroborate in acetonitrile.

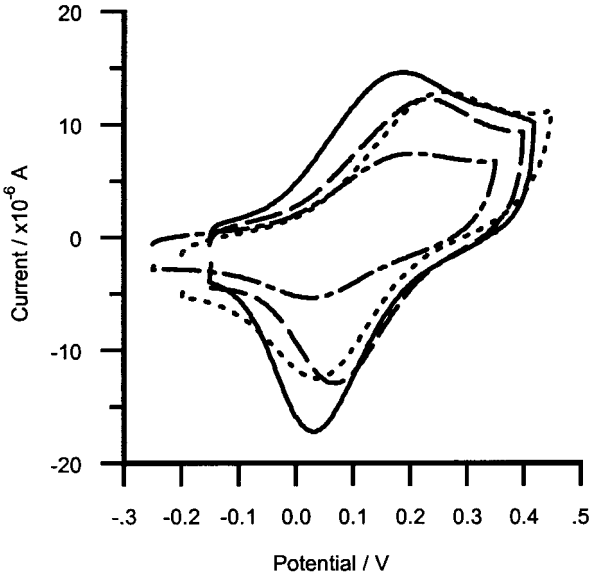

Fig. 9 Cyclic voltammograms of self-assembled films of Fcb-16Cys2X on evaporated gold slides. Dot–dash line: peptide-disulfide (X=S_2) self-assembled from acetonitrile; dotted line: disulfide (X=S_2) self-assembled from methanol; dashed line: dithiol (X=(SH)$_2$) self-assembled from ethanol; continuous line: dithiol (X=(SH)$_2$) self-assembled from methanol. All CVs were recorded at 80 mV s^{-1} in 0.1 M aq. $NaClO_4$ *vs.* a Ag wire (Ag/Ag$^+$) reference.

this and the measured anodic charges, which were now significantly greater than calculated for a monolayer of **6**, we concluded that the adsorbed film was several layers thick. A reasonable explanation for this solvent-dependent voltammetry is that the hydrophobic peptide was solvated better by the organic than by the aqueous solvent, allowing greater permeation of the (smaller) electrolyte counter-ion into the adsorbed film and lowering the activation energy of the $Fc^{0/+1}$ oxidation.

Films of the ferrocene derivative of **6**, disulfide and dithiol were also prepared on evaporated gold that was immersed in dilute peptide solutions for 5 days. The adsorbed layer was characterised by ellipsometry to measure the thickness and then by CV to measure the surface coverage. The voltammograms, which clearly feature the ferrocene wave, are shown in Fig. 9 and the calculated surface coverage and changes in the ellipsometric angles are summarised in Table 1. As can be seen from the Table the films prepared from ethanol or methanol

Table 1 Thickness and coverage of self-assembled films of Fcb-16Cys2 on evaporated gold slides, calculated from ellipsometry and cyclic voltammetry

Sample	$\delta\Delta^a$	$\delta\Psi^a$	Surface coverage (%)^b
Dithiol 230 μM MeOH	28.4	−2.3	294
Dithiol 80 μM EtOH	9.5	−1.0	101
Disulfide 210 μM MeOH	17.5	−1.1	216
Disulfide 90 μM MeCN	1.7	−0.1	90

$^a\delta\Delta=\Delta$(bare gold)$-\Delta$(coated slide), $\delta\Psi$ was calculated similarly. ^{b}Calculated from CV assuming that each peptide molecule covered 43×12 Å of the surface with a surface density of 1.9×10^{13} molecules cm^{-2}. A monolayer of peptide **6** would be 12 Å thick and compared to bare gold would decrease Δ by 1.5 and result in approximately no change in Ψ.

were characterised by ellipsometry as being several molecular layers thick, even though the measured charge of the peptide layer that self-assembled from ethanol was that expected for a monolayer. This apparent contradiction is reminiscent of the 'false' surface coverage measured of the film prepared on the gold disk electrode (described above) and probably has the same cause. This film was also characterised by RAIRS. The intensity of the bands in the surface spectrum (not shown) were consistent with a film of multilayer thickness, in agreement with ellipsometry.

In view of these results we concluded that even on a gold surface protic solvents could disrupt the peptide helix conformation sufficiently that one of the thiol groups became available to form a disulfide bond with a molecule in solution. The aggregation process could continue and lead to the build up of a thick film of oligodisulfide on the metal as observed. This hypothesis was given greater credence by the much more successful experiments carried out in non-protic acetonitrile.

The changes in the ellipsometric angles and charge passed for oligopeptide **6**, Fcb-16Cys2(S_2)-OH self-assembled from acetonitrile solution are shown in Table 1. Strikingly both sets of values are now in good agreement and correspond very well with those calculated for a close-packed SAM of helical peptide **6**. The successful preparation of monolayers of 16Cys2, therefore appears to require use of solvents which do not disrupt the hydrogen-bonding of the backbone peptide and thus stabilise the helix conformation. Interestingly this is exactly what was found in the earlier formation of the intramolecular S–S bond. Efficient preorganisation of binding sites and efficient self-assembly onto gold are therefore both key requirements for successful preparation of helical peptide monolayers.

Summary and prospects

We have outlined a generic strategy for preparing chiral functionalised surfaces using SAMs of helical oligopeptides. Those peptides containing Met are mainly helical in organic solvents and RAIRS of their SAMs on gold confirmed that they are aligned with the helix axes parallel to the surface. However these peptides are of low solubility which leads to handling problems. Their coverage and strength of binding to the surface are also variable probably because the weak sulfur–gold bond of thioethers means surface preparation is of utmost importance. Peptides with the thiol-containing Cys as the gold-binding residue also face potential problems. In particular there is a strong risk of oligomerisation occurring through formation of intermolecular disulfide bonds, either during S–S bond formation or during self-assembly to the gold. This is particularly prevalent in protic solvents and can lead to formation of thick films. However, if an aprotic solvent such as acetonitrile is used, the increased stabilisation of the helix and resultant preorganisation of the thiols allows efficient self-assembly of close-packed monomeric peptide monolayers. These monolayers offer a viable route for preparing functionalised surfaces with nanoscale spatial resolution. As such they are likely to find applications in fields such as molecular electronics, biosensors, biomaterials and catalysis.

Experimental

Peptide synthesis

General. Oligopeptides were synthesised by a Novasyn® Crystal Continuous Flow Peptide Synthesiser. Fmoc amino acids were activated with PyBOP® (benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate) and *N*-hydroxybenzotriazole (HOBt, one equivalent each) and diisopropylethylamine (DIPEA) (2 equivalents for Ala, Met, Fcb, Phe, (pNO_2)Phe, Leu and (Trt)Cys, 3 equivalents for Aib and Nle) immediately prior to injection onto the reaction column. A column temperature of 30 °C was maintained. Each residue was double coupled with a recirculation time of 40 min using a 2.5-fold excess of Fmoc-amino acid (Fmoc = fluorenylmethoxycarbonyl) for 12Leu2 and a 3-fold excess for all other peptides. Acylations were monitored at 348 nm. Fmoc deprotection by 20% piperidine in DMF (dimethylformamide) for 15 min was followed at 300 nm. Deprotection of the resin-bound Fmoc (PR500 amide resin) or pre-coupled Fmoc-Ala (PA500 acid resin) was carried out twice.

HPLC was carried out on analytical (250 × 4.6 mm) and semi-prep. (250 × 10 mm) columns each containing 5 μm C8 Spherisorb stationary phase. Flow rates were 1 ml min^{-1} for the analytical and 5 ml min^{-1} for the semi-prep. column and detection was at 230 nm unless stated otherwise. Elution was by gradients of water with either methanol or DMF (see supplementary information).

Electrospray ionisation mass spectra (ES-MS) were acquired on a Fisons VG Platform. The carrier solvent was 1 : 1 acetonitrile–water. Circular dichroic spectra were measured using a Jasco J600 spectropolarimeter with a 1 cm path-length cell. Molecular models were constructed on a Silicon Graphics Iris workstation using the M. S. I. software packages Builder and Biopolymer.

Materials. All commercial samples used in this study were used as received. All resins, Fmoc-amino acids and coupling reagents were of peptide synthesis grade and were supplied by Novabiochem. Acetic anhydride (98%), acetonitrile (for CD, spectrophotometric grade), *tert*-amyl alcohol (99%), carbon tetrachloride (99%), DBU (98%), dichloromethane (reagent grade), DIPEA (99%), lithium bromide (99+%), methanol (for CD, spectrophotometric grade), *p*-nitrobenzoyl chloride (NB-Cl, 98%) and triisopropylsilane (TIPS, 99%) were purchased from Aldrich. *N*,*N*-Dimethylformamide (DMF), diethyl ether, piperidine and trifluoroacetic acid (TFA) were all of peptide synthesis grade and were supplied by Rathburn. Acetonitrile and methanol used for HPLC were HPLC grade and were supplied by Merck. Glacial acetic acid and iodine (99.5+%) were supplied by Fisons. 4-Ferrocenylbutyric acid and 3-ferrocenylpropionic acid were gifts from Dr P. Whittaker, University of Strathclyde.

Resin washing protocol. The protected peptide-resin was washed sequentially with DMF, *tert*-amyl alcohol, glacial acetic acid, *tert*-amyl alcohol, dichloromethane and diethyl ether, dried under high vacuum for at least 5 h and stored under nitrogen at −20 °C.

Post-synthesiser deprotection. Protected peptide-resin (22 μmol of peptide) was swelled in DMF (4 ml) for 1 h. Piperidine was added to a ratio of 25% and the mixture was agitated gently. After 5 min standing the resin was filtered under gravity, washed twice with DMF and stored with 25% piperidine in DMF for 10 min, swirling occasionally. The resin was filtered under gravity, washed three times with DMF and acylated immediately.

Post-synthesiser acetylation. Acetic anhydride (30 μl, 0.3 mmol) and DIPEA (40 μl, 0.2 mmol) were added to the deprotected peptide-resin (40 μmol of peptide) in DMF (4 ml). After occasional gentle swirling for 30–45 min the resin was filtered and washed twice with DMF. Fresh DMF, acetic anhydride and DIPEA were added to the resin and the acylation was repeated. The Ac-peptide-resin was filtered, washed by the same protocol as the post-synthesis washing then either dried under high vacuum and stored under nitrogen at −20 °C or the peptide was cleaved immediately.

Post-synthesiser acylation with 4-ferrocenylbutyric acid. Deprotected peptide-resin (22 μmol of peptide) was treated

twice with a solution of 4-ferrocenylbutyric acid (18 mg, 66 μmol), PyBOP® (34 mg, 66 μmol), HOBt (10 mg, 66 μmol) and DIPEA (35 μl, 200 μmol) in DMF (5 ml) for 30–45 min. The resin was filtered and washed twice with DMF between couplings. After the second acylation the resin was washed and dried as described in the acetylation procedure.

Post-synthesiser acylation with *p*-nitrobenzoyl chloride. Swelled, deprotected peptide-resin (3 μmol of peptide) was acylated by double-coupling for 45 min with *p*-nitrobenzoyl chloride (3 mg, 16 μmol) and DIPEA (5 μl, 25 μmol) in DMF (0.5 ml). The peptide-resin was washed twice between couplings with DMF and after the second wash was treated in a similar manner to the acetylated peptide-resin.

Standard cleavage procedure for amide resin. Ac-peptide-resin (26 mg, 9 μmol of peptide) was swelled by washing on a frit with glacial acetic acid, dichloromethane and methanol (4 ml each). The resin was transferred to a flask and a solution of 10% TFA in dichloromethane (total 10 ml) was added. The beads turned red immediately. The mixture was swirled gently every 10–15 min for 45 min, the resin was filtered and the beads were stored in fresh cleavage mixture for a further 45 min. The resin was filtered again and washed with 10% TFA in dichloromethane (2×10 ml) and once with methanol (10 ml). The combined filtrates were diluted with a volume of acetonitrile equal to that of TFA and concentrated under reduced pressure to an oil. This was triturated with diethyl ether (16 ml) and the resulting white precipitate was centrifuged (3×20 min). The collected solid was either dried under high vacuum overnight and stored under nitrogen at $-20\,^{\circ}C$ or used immediately to prepare SAMs.

Standard cleavage procedure for acid resin. Triisopropylsilane (TIPS, 13 μl, 66 μmol), methanol (0.25 ml) and TFA (4.75 ml) were added to peptide-resin (82 mg, 16 μmol of peptide). The mixture was shaken occasionally and after 1–2 h was filtered. The resin was washed with 95:5 TFA–methanol (2×5 ml) and 1:1 TFA–methanol (5 ml). The combined filtrates were concentrated, precipitated with diethyl ether, centrifuged and dried according to the same protocol as used for the amide resin.

Protocol for oxygen-free cleavage. Peptide-resin was transferred to a fritted column equipped with a tap at the bottom and a nitrogen inlet at the top. The apparatus was charged with nitrogen and the resin was washed sequentially with acetic acid, dichloromethane and methanol. Degassed TFA and dichloromethane or methanol were added and the column was sealed under a nitrogen atmosphere, gently agitating the column every 10–15 min. After 45 min the solvents were filtered, freshly degassed cleavage solution was added and the mixture allowed to stand for a further 45 min. The solvents were filtered and the resin was washed with cleavage mixture and with methanol. The solution of cleaved peptide was removed *via* syringe and concentrated under reduced pressure to an oil. This was subsequently treated in the manner described in the standard cleavage procedure.

Peptide purification and characterisation. Available as supplementary material.

Preparation and characterisation of self-assembled monolayers

Materials. Absolute alcohol (reagent grade), hexaammineruthenium(II) chloride, sodium tetrafluoroborate (98%) and tetra-*n*-butylammonium tetrafluoroborate (99%) were supplied by Aldrich. Acetone, hydrogen peroxide (30 vol.%) and conc. hydrochloric, nitric and sulfuric acids were all reagent grade and were supplied by Fisons. Potassium chloride (AnalaR®) and sodium perchlorate (98%) were supplied by Merck. Acetonitrile (HPLC grade) was puchased from Rathburn. Chromium tris(tetrachlorocatecholate) was prepared by the method of Pierpont *et al.*[63]

Substrate preparation. Gold disk electrodes (2 or 3 mm diameter, supplied by Oxford Electrodes) were manually polished with alumina paste, sonicated in water and dried gently in a stream of nitrogen. The electrode was immersed in 50% *aqua regia* (4:3:1 water–conc. HCl–conc. HNO_3) for 60 s, water for 10 s, the contacting solvent for 10 s and then the peptide solution. When the contacting solvent was dichloromethane the electrode was dipped in methanol for 10 s between the water and dichloromethane rinses. Electrodes prepared in this manner were measured to have surface roughness of 2, from the anodic oxidation of adsorbed iodine to aqueous iodate.[64]

Evaporated gold substrates were prepared on glass microscope slides (from Merck) for RAIRS of peptides **1** and **2**, silicon wafers (100 orientation, from Micro-Image Technology Ltd.) for peptides **3** and **4** and on polyacrylamide gel slabs (from Pharmacia) for **6**. The glass microscope slides were cut to squares of approx. 2.5 cm, cleaned in piranha solution (1:1 H_2O_2–conc. H_2SO_4 **CAUTION!** THESE SOLUTIONS ARE HIGHLY OXIDISING AND SHOULD BE HANDLED WITH EXTREME CARE!) for 1 h, washed thoroughly with distilled water and sonicated for 20 min each in acetone and absolute ethanol. The slides remained immersed in ethanol until they were placed in the evaporating chamber, where 3–5 nm of chromium and 40–50 nm of gold were evaporated at a base pressure of 2×10^{-6} Torr and were immediately transferred to the contacting solutions.

The other gold slides were prepared under semi-conductor technology clean room conditions. Polyacrylamide gel slabs were cut into strips 20×70 mm and placed on an Al mask so that the coated area was of 2 rectangles 18×16 mm connected by a strip 2 mm wide and 28 mm long. Si wafers were either used whole or cut into strips 8 mm wide. The substrates were cleaned by sonication in either IPA or water–detergent then in water and dried in a stream of high-purity N_2. Cr and Au were evaporated at a rate of 1 Å s^{-1} to thicknesses of 100 and 1000 Å. The slides prepared on silicon wafers were immersed in piranha solution for 45 min, rinsed thoroughly with distilled water and dried in a stream of N_2. All other slides were used without further treatment.

Preparation of peptide and amino acid films. All solutions of peptides **3**, **4** and **6** were degassed except for the SAM of **6** on a gold disk electrode. After immersion the substrates were rinsed to remove excess peptide, unless stated otherwise in the text.

Electrochemistry

CV was controlled by an Autolab® potentiostat from Eco Chemie *via* General Purpose Electrochemical System 3 on a PC. A one-compartment cell was used. Oxygen was not excluded. The counter electrode was a coiled Pt wire, supplied by Goodfellow. The reference electrodes used were Ag/AgCl (Oxford electrodes) for voltammetry of peptides **1** and **2**, a Ag wire coated in AgCl (a gift from C. Agra-Gutierrez) for CV of $Ru(NH_3)_6Cl_2$ and $K_3[Cr(Cl_4C_6O_2)_3]$ and a silver wire (Ag/Ag^+) for all other experiments. The electrolytes were 10 or 100 mM tetra-*n*-butylammonium tetrafluoroborate in organic solvents, saturated KCl in 9:1 methanol–water for $K_3[Cr(Cl_4C_6O_2)_3]$ and aqueous solutions of 0.3 M KCl for electrochemistry of $Ru(NH_3)_6Cl_2$, 0.1 M $NaClO_4$ or 0.1 M $NaBF_4$.

Reflection–absorption infrared spectroscopy (RAIRS)

Infrared spectra were recorded on a Unicam Galaxy Series FTIR 7000 equipped with a liquid nitrogen-cooled Hg–Cd–Te detector and an FT85 specular reflector from Spectra Tech Inc. The sample compartment was purged with dry air. The angle of incidence was at 85° to the surface normal and the incident radiation was plane-polarised parallel to the direction of propagation. Reflection–absorption spectra were accumulated over 200 scans at 4 cm^{-1} resolution and were ratioed against an unused gold slide or a control slide that had been immersed in the same solvent.

Ellipsometry

Ellipsometry measurements were made on a PC-controlled Auto Gain Ellipsometer L116B from the Gaertner Scientific Corporation, Chicago. The light incident at 70° to the normal was at a wavelength of 632.8 nm from a He–Ne laser. The optical properties of the substrate and of the adsorbed layer were calculated using the classical 2- and 3-phase parallel layer models. The real component of the refractive index of the films, n_F, was assumed to be 1.500. The average of four recordings were made at each of three positions per slide. The surface area sampled by the laser was 2 mm^2. The optical characteristics of the bare gold were calculated from the measurements made on the same slide before immersion in solution.

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