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Exploring the use of the tripeptide Gly–Gly–His as a selective recognition element for the fabrication of electrochemical copper sensors

Wenrong Yang, Edith Chow, Gary. D. Willett, D. Brynn Hibbert and J. Justin Gooding*

School of Chemical Sciences, The University of New South Wales, Sydney 2052, Australia. E-mail: Justin.gooding@unsw.edu.au

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The modification of electrodes with the tripeptide Gly–Gly–His for the detection of copper in water samples is described in detail. The tripeptide modified electrode was prepared by first self-assembling 3-mercaptopropionic acid (MPA) onto the gold electrode followed by covalent attachment of the tripeptide to the self-assembled monolayer using carbodiimide coupling. The electrodes were characterized using electrochemistry, a newly developed mass-spectrometry method and quantum mechanical calculations. The mass spectrometry confirmed the modification to proceed as expected with peptide bonds formed between the carboxylic acids of the MPA and the terminal amine of the peptide. Electrochemical measurements indicated that approximately half the MPA molecules in a SAM are modified with the peptide. The peptide modified electrodes exhibited high sensitivity to copper which is attributed to the stable 4N coordinate complex the peptide formed around the metal ion to give copper the preferred tetragonal coordination. The formation of a 4 coordinate complex was predicted using quantum mechanical calculation and confirmed using mass spectrometry. The adsorption of the copper to the peptide modified electrode was consistent with a Langmuir isotherm with a binding constant of $(8.1 \pm 0.4)\ 10^{10}$ M^{-1} at 25 °C.

Introduction

The development of practical sensors for the detection and quantification of metal ions in environmental samples is the subject of considerable research. The majority of this research involves the synthesis and testing of macrocyclic ligands with selectivity for a target metal ion. However, in nature high selectivity for the binding of specific metals is achieved using peptide motifs. As a consequence there is a growing interest in exploiting this selectivity for analytical purposes using both fluorescence measurements[1–4] and modified electrodes.[5–10]

There are a number of attractive features of using peptides in the development of electrochemical metal ion sensors. First, many examples of highly selective metal binding peptide motifs are available from the protein literature. Secondly, the range of naturally occurring and synthetic amino acids available serves to provide the building blocks from which a great number of different metal ion selective ligands can be synthesized. Thirdly, similar synthetic strategies can be used whatever the required peptide sequence. The implications of synthesizing the selective ligands composed of different building blocks with generic chemistry is that it becomes relatively simple to tune the selectivity of the ligand for a given metal. Finally, if a generic method of attaching peptide sequences onto an electrode can be developed, then the same basic technology can be used to fabricate a sensor for any metal. It is exploitation of peptide ligands and the establishment of generic immobilisation technologies for the development of electrochemical metal ion sensors which is the focus of our research.

To create an oriented layer of peptide molecules at a surface requires suitable attachment chemistry with control over the conformation and spacing of the molecules to give a packing density that allows the analyte to access the binding sites. Recently we communicated the application of electrodes modified with the tripeptide Gly–Gly–His as copper ion sensors.[9] The electrodes were shown to have remarkably low detection limits and good selectivity for copper. An important aspect of this work was the interface to which the peptide was attached. The electrode was first modified with a self-assembled monolayer (SAM) of 3-mercaptopropionic acid (MPA). This carboxylic acid terminated SAM was then activated using carbodiimide chemistry in the presence of a succinimide ester[11,12] to give a SAM terminated by a succinimide ester. The succinimide ester terminated SAM was now susceptible to nucleophilic attack from an amine, such as that on the terminus of the tripeptide, to give a covalently attached molecule. This represents a generic strategy for preparing peptide and other molecule modified surfaces.[8,13] Similar approaches have been used by Frey and Corn[12] where poly(L-lysine) was attach to mercaptoundecanoic acid and Miura *et al.*[14] where ion pairing was used to attach helical peptides to a SAM of 11-mercaptoundecanoic acid. The key advantage of using MPA for electrochemical sensing is that the short three-carbon chain leads to a disordered SAM that allows the peptide ligand to be immobilised on a metal surface that is still electrochemically accessible.[13] The purpose of this paper is to characterise the Gly–Gly–His modified electrodes in more detail so as to confirm the proposed immobilisation of the peptide, the optimal surface chemistry and the mode of metal ion complexation.

Experimental section

Reagents and materials

3-Mercaptopropionic acid (MPA), mercaptopropane (MP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), Gly–Gly–His, 2-(*N*-morpholino)ethanesulfonic acid (MES), cadmium (II) chloride and mercury (II) chloride were obtained from Sigma Chem. Co. (Sydney, Australia). Reagent grade dipotassium orthophosphate, potassium dihydrogen orthophosphate, potassium chloride, sodium chloride, sodium hydroxide, sodium acetate,

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sodium bromide, sodium nitrate, silver nitrate, perchloric acid, cobalt (II) nitrate, calcium (II) nitrate and ethanol were purchased from Ajax Chemicals Pty. Ltd. (Sydney, Australia). Copper (II) nitrate and nickel (II) nitrate were from Prolabo (Paris, France). Ammonium acetate, lead (II) nitrate and zinc (II) nitrate were from APS Finechem (Sydney, Australia). Barium (II) nitrite was from Fluka (Sydney, Australia) and iron (III) nitrate was from May & Baker (Sydney, Australia). All reagents were used without further purification. All solutions were prepared with purified water (18 MΩ cm, Millipore, Sydney, Australia). Buffer solutions used in this work were 0.05 M K_2HPO_4/KH_2PO_4 in 0.05 M KCl and 0.05 M ammonium acetate. The pH was adjusted with either NaOH or HNO_3 solutions.

Glassware was soaked in 6 M HNO_3 and carefully cleaned before use to avoid contamination by metal ions.

Electrochemical measurements

All electrochemical measurements were performed with a BAS-100B electrochemical analyser (Bioanalytical System Inc., USA) and a conventional three-electrode system comprising a bare or modified working electrode, a platinum foil as the auxiliary and an Ag|AgCl|3.0 M NaCl electrode (from BAS) as the reference. All potentials are reported *versus* this reference at room temperature. The solution was degassed with N_2 for approximately 20 min prior to data acquisition and was blanketed with a N_2 atmosphere during the entire experimental period.

Cyclic voltammetry was conducted at a sweep rate of 100 mV s^{-1} between −200 mV and +600 mV. In Osteryoung square wave voltammetry the pulse amplitude was 25 mV with a step of 4 mV and frequency of 15 Hz. OSWV voltammograms were measured between −200 mV and +400 mV.

The electrochemical surface area of the gold electrode was determined from the charge passed during electrochemical oxidation in 1 M sulfuric acid as 0.020 ± 0.002 cm^{-2}.

Preparation of modified electrodes

Bulk gold electrodes were prepared by sealing polycrystalline gold wire (> 99.99% gold, Goodfellow, Cambridge, UK) in glass tubes followed by attaching copper wires for electrical connection to the back of the electrode. The electrodes were first polished to a mirror-like finish with 1.0 μm alumina, followed by 0.3 μm and 0.05 μm alumina slurry on microcloth pads (Buehler, Lake Bluff, IL, USA). After removal of the trace alumina from the surface by rinsing with water and brief cleaning in an ultrasonic bath, the electrodes were cleaned by electrochemical etching in 0.1 M H_2SO_4 by cycling the electrode potential between −300 mV and +1500 mV until a reproducible cyclic voltammogram was obtained. Modification of the electrodes was performed as outlined in Scheme 1. First, an MPA monolayer was prepared by immersing the gold electrode in 1 mM MPA ethanolic solution for 12 h, followed by rinsing with absolute ethanol. The gold electrodes were transferred to an MES buffer solution (pH 6.8) containing 15 mM NHS and 75 mM EDC for 30 min. The resultant NHS ester monolayers were reacted for 30 min in a solution of Gly–Gly–His (50 mg mL^{-1}) in MES buffer. All the modifications were performed at room temperature.

Mixed SAMs

Mixed SAMs comprising MPA and 3-mercaptopropane (MP) were prepared by immersing the gold-coated substrates in solutions of MPA and MP mixtures of a given fraction overnight; the gold electrodes were then removed, rinsed with ethanol, and dried under a stream of nitrogen. The mole ratios of all the SAMs reported in this paper refer to the mole fraction of the solutions used to prepare the SAM. On the basis of earlier studies,[15–17] it was suggested that the surface composition of the SAMs would be similar to but not necessarily identical to the solution composition. However by adjusting the solution composition of different alkanethiols the composition of the surface can be altered.

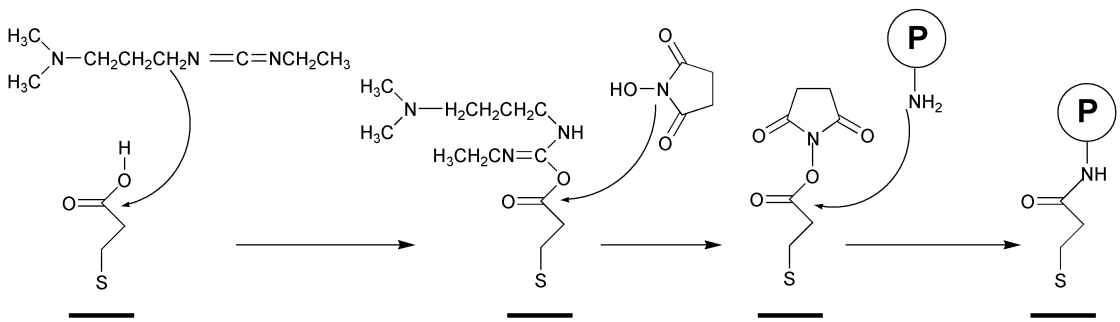
Measurement procedure

Copper ions were accumulated at the Gly–Gly–His modified electrode at open circuit by dipping the electrode into 10 mL of a stirred, buffered solution of copper nitrate for a given time (the accumulation time). The electrode was removed from the solution and washed thoroughly with purified water. It was then transferred to a cell containing a copper-free ammonium acetate buffer solution (pH 7.0). Cyclic voltammetry (CV) and Osteryoung square wave voltammetry experiments (OSWV) were conducted immediately. Regeneration of a copper-free electrode involved the elimination of Cu(II) by holding the working electrode at +0.5 V for 30 s in 0.1 M $HClO_4$.

Interference studies were performed by immersing an electrode in a solution of 9.8 nM Cu^{2+} (nominal concentration) plus another ion, the concentration of which was increased in decades until a measurable change in the redox current of copper was detected (> 0.5%) in difference OSW voltammetry.

FT-ICR MS experimental conditions

All experiments were conducted on a commercial Bruker BioAPEX 70e (Billerica, MA) Fourier transform ion cyclotron



Scheme 1 Schematic of the preparation of Gly–Gly–His modified electrodes on polycrystalline gold. Self-assembly of MPA was achieved by soaking the electrodes in a 1.0 mM MPA solution in ethanol for 12 h. The carboxylic acid terminated SAM was activated using EDC/NHS. The resultant succinimide ester monolayers were reacted for 30 min in a solution of Gly–Gly–His (50 mg mL^{-1}) in MES buffer to give a modified electrode shown where the tripeptide is attached through the amino group of the first glycine and terminates with the carboxylic acid of the histidine.

resonance mass spectrometer (FT ICR MS) controlled by Bruker XMASS software. Samples were introduced into the electrospray ionisation (ESI) source with a Cole-Parmer Series 74900 infusion pump at a rate of 60–90 μL h^{-1} by a syringe that contains the sample solution at a concentration of 100 μg L^{-1}. Prior to the mass analysis of the peptide, the FT ICR MS was mass-calibrated using sodium iodide cluster ions formed by ESI from a sodium iodide methanol solution at a concentration of 6.7×10^{-4} M (0.1 mg mL^{-1}). To characterise peptide modified surfaces using mass spectrometry a procedure described elsewhere was used.[18] In brief, the SAM was reductively desorbed by scanning the electrode potential −400 and −1200 mV *versus* Ag/AgCl at 100 mV s^{-1} in a stirred 1 mL 0.1 KOH solution[19–21] which contained 0.2 mg octadecyl derivatized silica gel (C_{18}SG) (particle size: 40 μM) in 0.2 ml 50% acetonitrile in water. The peptide accumulated on the C_{18}SG which was subsequently filtered with a Durapore membrane and rinsed with Milli-Q water. The peptide was eluted from the C_{18}SG using two aliquots of 0.5 mL mixture of methanol and water (4:1 v/v). The eluted solution was then analysed by FT ICR MS in negative ion mode. The negative ion spectrum was preferred because previous studies have shown that the positive ion mass spectrum of SAMs contain few structurally specific molecular species.[22–24]

All quantum mechanical calculations were performed on a Pentium III 733 (128 MB, 20 GB) computer using HyperChem 6.0.[25] The geometry optimization calculations of peptide–Cu complexes were performed by using the Polak-Ribiere algorithm in the RHF-SCF-PM3 method.[26]

Results and discussion

The electrode interface

An important aspect of using peptide modified electrodes as a generic strategy for the detection of metal ions is that the sensing interface is fabricated in the same manner regardless of the peptide used and the metal to be detected. The interface used in this study, as outlined in Scheme 1, fulfils that criterion but there are a number of steps involved in the fabrication. These steps involve preparing the gold surface, assembling MPA onto the surface, activating the MPA and finally attaching the peptide. Normally, the success or failure of any of these steps is only evaluated once the peptide is attached and the electrode is exposed to the metal ion. Fig. 1 shows a cyclic voltammogram at a sweep rate of 100 mV s^{-1} of the peptide-modified electrode before and after incubation in a copper solution. The electrochemistry due to the reduction of Cu(II) to Cu(I) and the oxidation back to Cu(II) suggests the peptide is associated with the interface. If the peptide is absent, such that the electrode is only modified with MPA, there is no response in the CV or the more sensitive OSWV to copper at 17 ppb (0.27 nM) (data not shown). Despite the inference that the peptide is attached to the electrode, it is still important to assess the reliability of each of these steps and to verify that the proposed covalently-attached peptide interface is in fact achieved.

The effect of the nature and preparation of the gold surface on the amount of MPA attached was investigated previously.[20,27] On bulk polycrystalline gold, as used in this study, the amount of MPA adsorbed was determined by reductive desorption of the SAM to be 0.62 ± 0.33 nmol cm^{-2} (n = 9). The high standard deviation implies an irreproducible assembly of the SAM onto the electrode. However, using MPA SAMs for the fabrication of enzyme electrodes, a variability of less than 10% has been reported for the immobilization of enzymes.[28] The high standard deviation here is attributed to the determination of surface coverage from the reductive desorption measurement. With adsorbed short chain alkanethiol the electrode is poorly passivating, such that the potential required to desorb the SAM coincides with other reduction processes occurring at the gold surface. This makes baseline subtraction difficult. In the case of a hexadecane SAM for which the electrode is effectively passivated and baseline subtraction is more straightforward, excellent reproducibility of the surface coverage was achieved (RSD of 6.8%).[20] Further evidence for good reproducibility of SAM formation is from our previous report of Gly–Gly–His modified electrodes when the response to copper showed a relative standard deviation in the linear region of the calibration curve of 4.8%.[9]

The activation of the MPA SAM using EDC and NHS has been well characterized using X-ray photoelectron spectroscopy.[29] Therefore, the next step in the characterization of the preparation of the peptide recognition interface is the covalent attachment of the peptide. Attachment of the peptide to the gold electrode is inferred from Fig. 1. To verify that the tripeptide is actually covalently attached to the SAM rather than just adsorbed at the interface as implied in Scheme 1, a novel mass spectrometry procedure was developed as described above and previously.[18] The negative ion mass spectrum obtained for a Gly–Gly–His modified electrode is shown in Fig. 2. The spectrum shows a prominent parent peak at 711.12 m/z with very little other fragmentation. The peak at 711.12 m/z is assigned to a disulfide dimer of Gly–Gly–His attached to MPA as shown in the inset of Fig. 2. This species can only have resulted from the covalent attachment of the tripeptide to the MPA SAM *via* the formation of a peptide bond. Importantly, the spectrum shows no evidence of Gly–Gly–His by itself at m/z of 268.11. When a solution of Gly–Gly–His was exposed to the C_{18} silica gel subsequent elution resulted in its detection by the mass spectrometer. Therefore the absence of the Gly–Gly–His peak shows that any non-specifically adsorbed peptide is removed during the preparation of the electrode prior to exposure to copper. It follows that the voltammogram in Fig. 1 is from copper complexed to covalently attached Gly–Gly–His.

The successful covalent attachment of Gly–Gly–His to the MPA modified electrode surface illustrates that a stable peptide modified interface can be fabricated which is applicable all

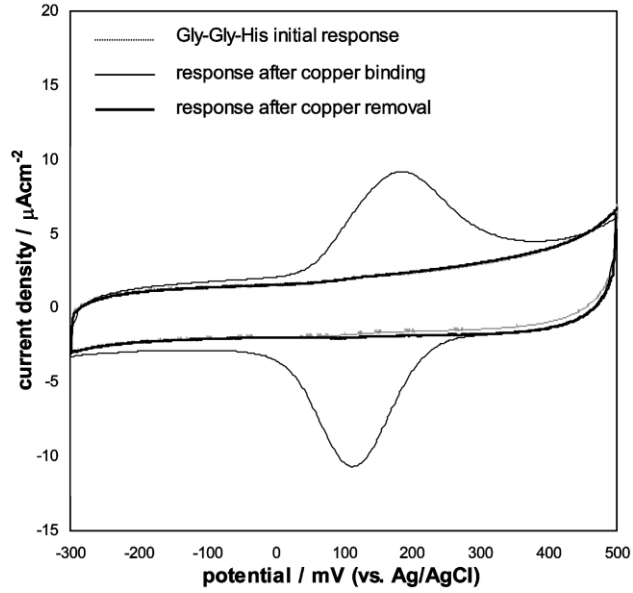


Fig. 1 Cyclic voltammogram of Gly–Gly–His modified electrode before and after exposure to copper in 0.05 M ammonium acetate buffer solution (pH 7.0). In all cases Cu(II) was accumulated at the Gly–Gly–His modified electrode at open circuit for 10 min in a 0.05 M ammonium acetate buffer solution (pH 7.0) containing 9×10^{-9} M copper nitrate, removed, rinsed and then placed in a copper-free ammonium acetate buffer solution. Also shown is the CV after regeneration of the electrode by holding the working electrode at +0.5 V for 30 s in 0.1 M $HClO_4$. Sweep rate: 100 mV s^{-1}.

peptides. However, this knowledge does not provide any information on whether this is the optimal interfacial structure for the detection of metal ions. One issue of concern is that every MPA molecule that assembles on the gold electrode is a potential site of attachment of a peptide. Therefore it seems conceivable that overcrowding of the interface by Gly–Gly–His might block copper binding. To ascertain whether a high density of coupling points was detrimental to the electrode, the number of coupling points on the SAM surface was varied by preparing mixed monolayers of MP and MPA. As MP does not possess the carboxylic acid group peptides cannot be attached and so MP dilutes the surface concentration of peptides. A number of electrodes were prepared from solutions containing increasing mole fractions of MPA relative to MP. Note however that the mole fraction of the two components on the final SAM is not necessarily the same as the mole fraction of the two components in solution.[16,17,30] Each electrode was exposed to a saturating amount of copper (0.1 μM), transferred to a copper free solution and the CV measured. The charge passed in the oxidation and reduction of the surface bound copper gave the amount of copper complexed at the surface. Fig. 3 shows the variation in the amount of copper as a function of composition of the MPA/MP solution. It is evident that the amount of copper complexed, and hence the current sensitivity of the sensor, increases with the mole fraction of MPA before reaching a plateau at a mole fraction of about 0.5. Further increases in MPA have neither a positive nor negative effect on the amount of Cu(II) complexed. Therefore, because of the simplicity of preparation, 100% MPA SAMs were used in sensor preparation.

The variation in surface coverage of copper with SAM composition gives an upper bound for the amount of Gly–Gly–His immobilised on the SAM surface by assuming that every Gly–Gly–His binds a Cu(II) at saturation. With increasing MPA mole fraction the number of Gly–Gly–His molecules attached increases and reaches a plateau at 0.5 mole fraction of MPA. This occurs at 0.25 ± 0.03 nmol cm^{-2} Cu(II) which is approximately half the surface concentration of MPA in a 100% MPA SAM of 0.62 ± 0.33 nmol cm^{-2}. These two results suggest that despite up to 90% of MPA molecules being activated by EDC and NHS[29] the tripeptide attaches to only about half the surface adsorbed MPA molecules. The limitation for Gly–Gly–His coverage is presumed to be a steric effect.

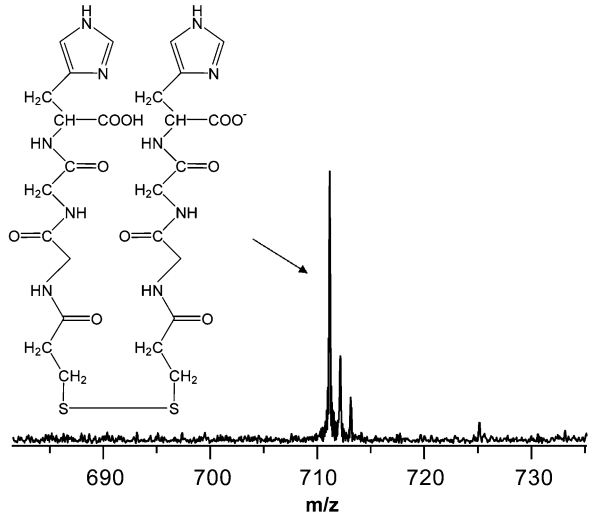


Fig. 2 The negative ion mass spectrum of the disulfide Gly–Gly–His desorbed from a gold electrode. (See the Experimental section).

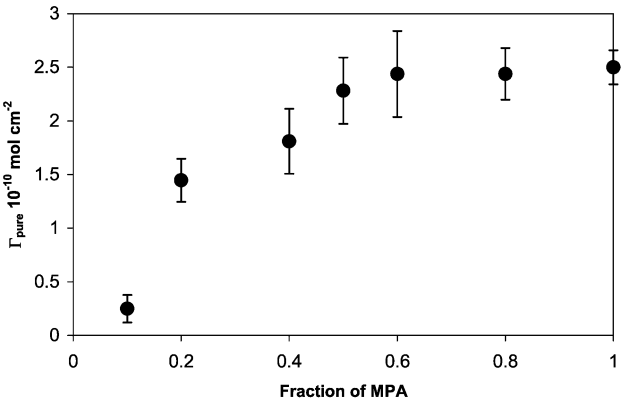


Fig. 3 Cu(II) coverage of Gly–Gly–His modified electrodes on a mixed SAM of MPA and MP Cu(II) coverage of the Gly–Gly–His modified gold electrodes (Γ), determined by integration of CV peaks. Mixed SAMs comprising MPA and MP were prepared by immersing the gold-coated substrates in solutions of mixtures of MPA and MP of a given fraction. In all cases Cu(II) was accumulated at the step-wise Gly–Gly–His modified electrode at open circuit for 10 min in a 0.05 M ammonia acetate buffer solution (pH 7.0) containing 0.1 μM copper nitrate, removed, rinsed and then placed in a copper-free ammonium acetate buffer solution. Scan rate: 100 mV s^{-1}.

The complexation of copper

The cyclic voltammogram in Fig. 1 shows the ability of the Gly–Gly–His modified electrodes to detect copper at low concentrations. However, OSW voltammetry was used for analytical measurements because of its greater sensitivity. In our previous work we published a calibration curve for copper with concentrations in the ppt (low pM) range.[9] It is important to emphasize that these concentrations are nominal as it is not possible to validate them as they are below the detection limit of ICP-MS. Considerable effort was taken to make these concentrations as accurate as possible. All glass containers were cleaned with distilled concentrated nitric acid, all solutions were made with Milli-Q water with copper levels below the detection limit of the ICP-MS, all solutions were made in a laminar flow cabinet to limit contamination from airborne copper. Electrode preparations and storage were performed in polypropylene containers and all measurements were performed in polystyrene cells. Solutions with copper concentrations in the ppb range were analysed by ICP-MS and gave values lower than the nominal value and therefore although we cannot independently assess the actual Cu(II) concentration at these levels we are confident there is no gross copper contamination. Furthermore, if there was gross copper contamination all the pM standards would be expected to give the same response.

The inability to validate the lowest copper concentrations measured by the electrode makes it worth considering whether such low detection limits are realistic. The current from OSWV is proportional to the amount of copper bound to the surface and therefore assuming a simple complexation equilibrium,

Au–MPA–Gly–Gly–His + Cu^{2+} (aq) ⇌ Au–MPA–(Gly–Gly–His–Cu^{2+})

eqn. (1) allows the determination of the binding constant (*K*, units: M^{-1}).

$$I_{OSWV} = -nF\frac{d\Gamma_{Cu}}{dt} = \frac{aKC_{Cu}}{1 + KC_{Cu}} \quad (1)$$

where *n* is the number of electrons in the redox process, *F* is the Faraday constant. The parameter *a* is a normalising constant with units μA cm^{-2}. Eqn. (1) is of the form of the Langmuir equation, being derived from similar assumptions of equilibrium and independence of sites.

Fig. 4 shows the best fit of eqn. (1) to the OSWV peak current against nominal copper concentration. A binding constant of (8.1 ± 0.4) × 10^{10} M^{-1} at 25 °C. The confidence interval is for

95% probability calculated from the Hessian of the sum of squares of residuals and variable matrix. This value for K is nearly half of the previously reported affinity constant of 1.3×10^{11} M^{-1} at 25 °C for Gly–Gly–His in solution,[31] which may be reasonable considering the loss in configuration freedom associated with the immobilization of the peptide. Furthermore, the fact that the affinity constant derived from the above analysis is less than the affinity constant for Gly–Gly–His in solution indicates that the low detection limits reported from the nominal concentrations are not inconceivable.

Quantum mechanical calculations

The relative binding energies of geometry-optimized tripeptides containing glycine and histidine for Cu(II) were calculated.[32] Gly–Gly–His forms a more stable complex than any other combination of tripeptides involving Gly and His (Table 1). The optimum geometry for Gly–Gly–His complexed with Cu(II) shows the metal has the required square planar coordination with nitrogen atoms from each peptide bond and the imidazole nitrogen of the histidine ring forming a 4N complex. This is not surprising as Cu(II) has a particularly high thermodynamic affinity for 4N chelating ligands.[33] Note that, as a result of the imidazole nitrogen being involved in the bonding of the copper, the carboxylic acid on the histidine ring is not involved in the metal complexation. Fragmentation of Gly–Gly–His in the presence and absence of copper as measured by collision induced dissociation (CID) using electrospray FTICRMS provides good evidence for the structure proposed. Negative ion spectra of the fragmentation of Gly–Gly–His in the absence of copper show the main fragments as cleavage of a histidine. In contrast when Gly–Gly–His is exposed to Cu(II) then the main fragment after collision induced dissociation is from the loss of a carboxylate. The carboxylate cleaved is the carboxylic acid from the histidine uninvolved in the metal complexation, see Scheme 2.

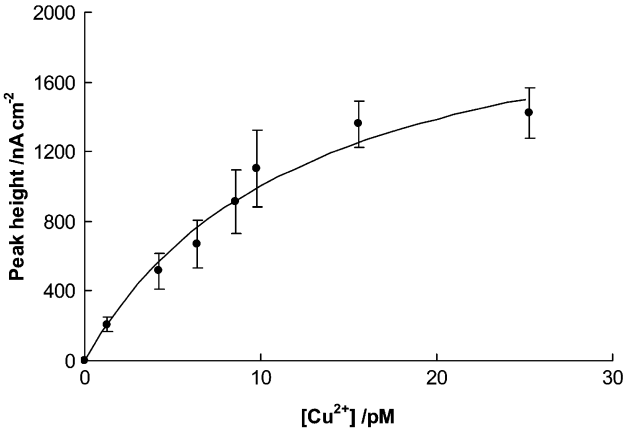


Fig. 4 Plot of I_{OSWV} *vs.* concentration of copper in solution. In all cases Cu(II) was accumulated at the Gly–Gly–His modified electrode at open circuit for 10 min in a 0.05 M ammonia acetate buffer solution (pH 7.0) containing 0.1 μM copper nitrate, removed, rinsed and then placed in a copper-free ammonium acetate buffer solution. The solid line is a fit to eqn. (1) (see text).

Table 1 Binding energies of different sequence peptides binding to Cu(II)[a]

Cu(II)	Binding energy/kJ mol^{-1}
Gly–Gly–His	−816.2
Gly–His–Gly	−769.8
His–His–His	−737.3
Gly–Gly–Gly	−560.7
Gly–His–His	−752.6
His–Gly–Gly	−762.6
His–His–Gly	−708.6

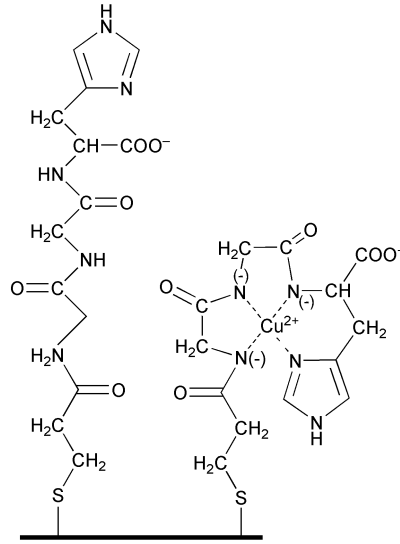
[a] All theoretical calculations of binding energies were performed on a Pentium III 733 (128 MB, 20 GB) computer using HyperChem 6.0. The geometry optimization calculations of peptide–Cu complexes were performed by using the Polak-Ribiere algorithm in the RHF-SCF-PM3 method.

Analytical performance

For Gly–Gly–His modified electrodes to be useful for routine analysis there are a number of factors which must be addressed other than the reproducibility of the electrodes as described above. These include: the selectivity of the electrode for copper, the ability to reuse the electrode, the stability of the electrodes and finally a successful demonstration of the analytical performance of the electrode.

Peptide modified electrodes have two levels of selectivity. The first derives from the affinity coefficient of a metal with the peptide and the second from the redox potential of the bound metal ion. An interfering species might cause a decrease in the current of the target metal by competitively occupying binding sites and it could also contribute current if its redox potential were sufficiently near that of the target metal. The selectivity of Gly–Gly–His modified electrodes for copper amongst Ni(II), Co(II), Pb(II), Ba(II), Cd(II), Ca(II), Fe(III), Zn(II) and Hg(II) has been described previously (see Table S1 in the electronic supplementary information of ref. 9). Apart from Ni(II), there was no interference until the metal ion concentration was micromolar or millimolar. Ni(II) is potentially the most serious interfering species because, like Cu(II), it forms square planar 4N complexes with peptides such as Gly–Gly–His in solution[34,35] and because it has a reduction potential which is close to that of Cu(II). The interference of Ni(II) however was only minor. When a Gly–Gly–His modified electrode was incubated in a solution 10 pM of both Cu(II) and Ni(II) there was only a 6.5% decrease in peak current compared to when Ni(II) was absent.

We next turn our attention to the regeneration and stability of the electrode. To reuse the electrode requires removal of all the accumulated copper from the Gly–Gly–His modified electrode. Modified electrodes used for Cu^{2+} analysis could be re-



Scheme 2 Schematic of the Gly–Gly–His modified electrode before and after copper binding.

generated by elimination of Cu^{2+} by holding the working electrode at +0.5 V for 30 s in 0.1 M $HClO_4$.[6] After regeneration the electrochemistry of Cu^{2+} completely disappears. The clean surface of the uncomplexed peptides is successfully restored, and the modified electrode is ready for further exposure to metal ions. The same electrode could be regenerated almost 20 times before significant deterioration of the signal was observed (Fig. 5a). Consequently, an electrode modified with peptides turns out to be a stable and reusable sensing probe. The storage stability of a Gly–Gly–His modified electrode was also assessed by storage under dry conditions at 4 °C. With this procedure the same electrode could be used every day for a week without loss of performance whereupon the current response began to decline (Fig. 5b). The loss of performance over time and repeated use is attributed to degradation of the SAM. SAMs of short chain alkanethiols are know to be oxidised in the presence of oxygen and light.[36–39] In the oxidation process the thiolate is converted to either a sulfinate or a sulfonate. In both cases the oxidised thiol is not as strongly adsorbed on the electrode surface as the thiol.[36] Therefore it is hypothesized that during the repeated use of the electrode the SAM is oxidised whereupon it is lost from the electrode surface.

To test the ability of a Gly–Gly–His modified electrode to analyse natural samples both tap water and a lake sample were analysed for copper. To perform these analyses the samples required dilution to reduce the copper concentration to within the range that the modified electrodes could analyse. The samples were also analysed using ICP-OES. The analytical results for the modified electrode and the ICP-OES are shown in Table 2. As can be seen good agreement is obtained between the electrode and the ICP-OES for both samples. The good agreement between the two methods for the lake sample shows that the peptide successfully competes with naturally occurring ligands, such as humic acids, for copper ions.

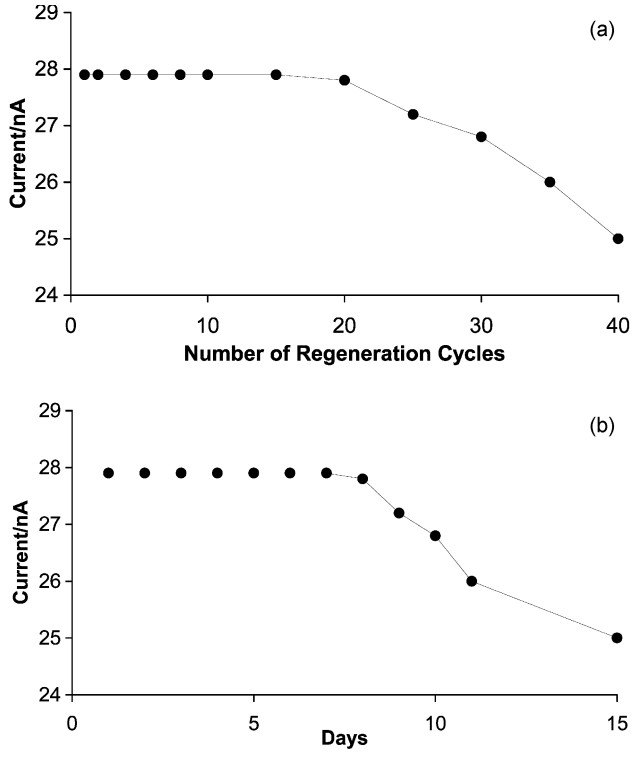


Fig. 5 (a) Plot of OSWV peak current *versus* the number of times that an electrode was regenerated after exposure to 9.8 pM of Cu(II) and (b) plot of the stability of Gly–Gly–His modified electrodes for the repetitive measurement of 9.8 pM Cu^{2+}. Modified electrodes used for Cu^{2+} analysis could be regenerated by elimination of Cu^{2+} by holding the working electrode at + 0.5 V for 30 s in 0.1 M $HClO_4$,[6,40] the then electrochemistry due to Cu^{2+} completely disappears. The clean surface of the uncomplexed peptides is successfully restored, and the modified electrode is ready for further exposure to metal ions. Furthermore, the same voltammetric behaviour is observed for several days without any deterioration.

Table 2 Analysis of Cu^{2+} in local water samples[a]

Sample	Gly–Gly–His modified electrode (ppm, standard deviation of 6 measurements)	ICP-MS (ppm, standard deviation of 3 measurements)
Tap water	0.27 (0.13)	0.33 (0.16)
Local lake water	88.75 (0.21)	86.51 (0.12)

[a] Analysis of a local tap water was performed by diluting the sample to bring the copper concentration to within the range of the Gly–Gly–His modified electrode. The measured concentration of free Cu^{2+} in the tap water, when dilutions were accounted for, was 0.27 ppm (s = 0.03, n = 3) which agreed well with the total copper concentration measured using inductively coupled plasma mass spectrometry (ICP-MS) of 0.33 ppm.

Conclusions

It has been demonstrated that the covalent attachment of Gly–Gly–His to a SAM on gold relies on the activation of carboxylic acid groups to NHS esters and the formation of an amide bond to the peptide. The optimal surface chemistry and the mode of metal ion complexation have been characterised in detail. These initial results illustrate the potential for oligopeptide-modified electrodes to be used as metal ion sensors with extraordinary low detection limits. Low detection limits coupled with good selectivity for Cu^{2+} satisfy the performance criteria for a single metal ion sensor.

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