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Extending the Dynamic Range of Peptide-Modified Electrodes for Detecting Copper(II) Ions by Using Multiple Modified Electrodes

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Abstract

Multiple electrodes have been used for the determination of copper ions across a broad dynamic range by attaching ligands possessing different binding affinities for copper to gold electrode surfaces modified with thiol self assembled monolayers . The electrodes were modified with the copper complexing species (3-mercaptopropionic acid, thioctic acid, and the peptides cysteine and Gly-Gly-His) and copper was determined over concentrations ranging from nanomolar to millimolar using voltammetric analysis. Thioctic acid is demonstrated to be a more stable thiol for the formation of a SAM. We have demonstrated that by combining the calibration functions from the four electrodes a better estimate (i.e. with smaller variance) of the concentration of the analyte is obtained. Measurement uncertainty is expressed for independently prepared electrodes, which allows the possibility of commercial production and factory calibration.

Keywords Metal detection, peptides, biosensors, electrochemistry, copper

Introduction

Copper is considered an essential trace element for humans with an estimated daily requirement of 2-3 mg [1]. However, elevated levels of copper can be toxic and can result in nausea, abdominal pain, vomiting and in extreme cases, death. The main sources of copper intake are through food and drinking water. This has led to government legislations defining upper guidelines levels for copper in drinking water (EU standard is 2.0 ppm (30 μM)) [2]. It is necessary to determine copper concentrations over a wide range of concentrations since copper in surface waters can be as high as several ppm in

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some circumstances even though concentrations less than 10 ppb are more common [1]. Since copper concentrations can vary to a large degree, one key goal in the analysis of this element is to be able to measure the target analyte at a wide range of possible concentrations. This is often one of the limitations of using the traditional approaches of inductively coupled plasma atomic emission spectroscopy (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) since they can only accurately determine elemental levels over a few orders of magnitude. The practical working range of ICP-AES is usually 0.1 ppm to several ppm and for ICP-MS this is limited to ~0.2 ppb to 1 ppm. Since the magnitude of copper in a sample can be unknown, it can be costly in terms of sample solution, time and money to carry out analyses using different instrumentation and/or using several dilutions.

The limitation of the classical approaches is also extended to metal ion sensors [3]. For example, Cu^{2+} electrochemical sensors using glycyl-glycyl-histidine (Gly-Gly-His) copper-binding peptide as the recognition element have successfully demonstrated the ability to measure Cu^{2+} concentrations as low as 0.2 ppt (3 pM) but the sensor's response is saturated at 1.6 ppt (25.3 pM) [4-6]. Similarly, we and other workers have used *L*-cysteine [7-9], poly-*L*-cysteine [10], poly-*L*-aspartate [11], glutathione [12] and 3-mercaptopropionic acid (MPA) [13, 14] for the detection of Cu^{2+} with different selectivities and sensitivities than Gly-Gly-His but with the same problem of a limited dynamic range. The limitation in dynamic range is a consequence of all these sensors having one or two binding modes to complex the metal ion. Biosensors for metal ions with significantly broader dynamic ranges have been described using proteins with multiple metal ion binding sites [15, 16]. The drawback of such metallothionein proteins was the multiple binding modes could bind many different metals; thus the biosensors suffered from poor selectivity for a given metal.

An approach towards increasing the dynamic range is to use an array of sensors of varying sensitivity and selectivity. In this paper we discuss strategies for using multiple electrodes to improve estimates of concentrations and errors, as well as extending the dynamic range of the sensor. The strategy employs four electrodes where each one is modified with a different ligand. The four ligands are thioctic acid (TA) [17, 18], MPA [13, 14], *L*-cysteine [7-9] and Gly-Gly-His [4-6]. In these experiments, metal ions are accumulated from solution at an electrode surface and the electrochemistry of the bound ion is studied by voltammetry. There have been some descriptions of using multivariate analysis for voltammetry of many ions, in which the overlap of peaks requires chemometric analysis in order to calibrate all species [19-21]. Here we concentrate on a single metal, but use multiple electrodes to refine the estimate of concentration and extend the range of applicable concentrations.

Moving away from linear calibration leads to challenges for use of the measurement function and for assigning a standard error to concentrations determined from it. If the calibration function can be inverted, and the change in slope is not too great across the calibration range, then a linear Taylor expansion of the variance of an estimated concentration can give a good estimate of the uncertainty. Although the mathematics is more complex than a simple linear calibration, the analysis can be done using proprietary software.

Theoretical Considerations: Calibration of Multiple Electrodes

The strategy to be employed here of four electrodes modified with self-assembled monolayers with different ligands for the same metal ion represents an extension of our previous work on using a single electrode modified with a suitable peptide for detecting a single element [4-7, 11, 12, 18, 22-25]. The selectivity of these peptide modified electrodes comes from the selectivity of the peptide for the equilibrium binding of certain metals. The metal is then detected amperometrically. The form of the current versus log(concentration) 'calibration curves' is typically an 'S'-shaped curve as expected for an equilibrium binding reaction. In our case we shall show the data is consistent with such a model and for some electrodes is complicated by requiring the combination of two binding modes, as the system evolves from tetradentate binding to bidentate as the concentration of the metal ion increases. When two binding modes exist the current against log(concentration) graph is a double step and it becomes impossible to discern suitable linear ranges. However by adopting a simple equilibrium model the data can be fitted over a wide range of concentrations and thus used for calibration:

$$I = \frac{I_0 K_{\mathrm{M}}[\mathrm{M}]}{1 + K_{\mathrm{M}}[\mathrm{M}]} \tag{1}$$

or

$$I = \frac{I_{0,1} K_{\mathrm{M},1}[\mathrm{M}]}{1 + K_{\mathrm{M},1}[\mathrm{M}]} + \frac{I_{0,2} K_{\mathrm{M},2}[\mathrm{M}]}{1 + K_{\mathrm{M},2}[\mathrm{M}]} \tag{2}$$

where I_0 and K_{M} are the saturation current and equilibrium constant for complexation respectively, and [M] is the concentration of the metal ion. Equations (1) or (2) can be fitted to calibration data and the parameters I_0 and K_{M} estimated, allowing inversion of the equation and solution for [M] given a measured current I.

$$[\mathrm{M}] = \frac{I}{(I_0 - I)K_{\mathrm{M}}} \tag{3}$$

from the rearrangement of Equation (1) or the positive root of the solution of

$$(I - I_{0,1} - I_{0,2})K_{\mathrm{M},1}K_{\mathrm{M},2}[\mathrm{M}]^2 + \left[K_{\mathrm{M},1}(I - I_{0,1}) + K_{\mathrm{M},2}(I - I_{0,2})\right][\mathrm{M}] + I = 0 \tag{4}$$

from Equation (2). Of importance is the uncertainty on a calculated value of [M] arising from the lack of fit of the chosen calibration equation and the measurement uncertainty of the current. Measurement models often assume constant variance in the observed variable, although data over a wide concentration range tends to an increasing standard deviation with concentration. With our data we will demonstrate that the standard deviation of the current is a function of concentration

$$s = a + b_1 \log_{10}([M]) + b_2 \{\log_{10}([M])\}^2 \tag{5}$$

with the parameters b_1 and b_2 being zero for some data. Massart *et al.* have argued that the most simple equation between reproducibility and concentration should be used to describe the relation [26]. The equation has no theoretical implications but is just a means of providing a function, which can be used in subsequent estimations of uncertainty, that follows the standard deviation reasonably well, and certainly better than a constant standard deviation or constant relative standard deviation.

When fitting data to Equations (1) and (2) the Jacobian of the fitted function yields the standard errors on the estimates of the parameters, and the variances and covariances of the parameters.

For an electrode that follows Equation (1) having fitted calibration data to obtain estimates of I_0 and K_M the concentration of the metal is given by Equation (3), and the variance of the estimated concentration, $V([M])$, arising from the least squares fit is

$$V([M]) = \left(\frac{\partial[M]}{\partial I}\right)^2 V(I) + \left(\frac{\partial[M]}{\partial I_0}\right)^2 V(I_0) + \left(\frac{\partial[M]}{\partial K_M}\right)^2 V(K_M) + 2\left(\frac{\partial[M]}{\partial K_M}\right)\left(\frac{\partial[M]}{\partial I_0}\right) Cov(K_M, I_0) \quad (6)$$

where $Cov(K_M,I_0)$ is the covariance between the parameters K_M and I_0. Evaluation of the differentials gives

$$V([M]) = \frac{I_0^2}{(I_0 - I)^4 K_M^2} V(I) + \frac{I^2}{(I_0 - I)^4 K_M^2} V(I_0) + \frac{I^2}{(I_0 - I)^2 K_M^4} V(K_M) + 2\frac{I^2}{(I_0 - I)^3 K_M^3} Cov(K_M, I_0) \quad (7)$$

Values of the parameters are known from the calibration and the variances and covariances are also obtained from the fit to calibration data. $V(I)$ can be taken from the standard error of the fit (in the same way that the linear calibration term is handled), or a pooled standard deviation of all measured currents, or from the square of a function of the form of Equation (5).

The uncertainty of a concentration estimated from the calibration equation is the square root of $V([M])$. The non-linearity of the calibration leads to non-Normal distribution of errors in the estimated concentration, so multiplication of $\sqrt{V([M])}$ by a Student t value to estimate a confidence interval may not be appropriate.

For the case of an electrode that follows Equation (2) the variance of an estimated concentration due to the fit is given by differentiation with respect to the four parameters ($I_{0,1}$, $I_{0,2}$, $K_{M,1}$, $K_{M,2}$) and six covariance terms (all two-term combinations of the parameters). The algebra is long, but straightforward, and can be accomplished using symbolic mathematics software.

Experimental Section

Four electrode modifications were chosen for study: gold electrodes modified with 3-mercaptopropionic acid (MPA), *DL*-6, 8-thioctic acid (TA), *L*-cysteine and the peptide glycyl-glycyl-histidine (Gly-Gly-His). MPA and TA are alkanethiols which have been used by the present authors to provide a self-assembled monolayer (SAM) upon which peptides [4-6, 11, 12, 18, 23], enzymes [27] and other molecules [9] are attached to give functional sensing systems on electrode surfaces. *L*-cysteine has a sulfur moiety that can self-assemble on gold [7] (see Figure 1 below).

Materials

Gly-Gly-His, *L*-cysteine, TA and *N*-hydroxysuccinimide (NHS) were purchased from Sigma (Sydney, Australia). MPA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(*N*-morpholino)-ethanesulfonic acid (MES), barium(II) nitrate, zinc(II) nitrate and perchloric acid were from Aldrich (Sydney, Australia). Sodium hydroxide, potassium hydroxide, sodium chloride, sulfuric acid, nitric acid, ammonium acetate, ethanol, copper(II) sulfate, lead(II) nitrate and chromium(III) nitrate were obtained from Ajax (Sydney, Australia). Cadmium(II) nitrate was purchased from Fluka (Sydney, Australia). Nickel (II) nitrate was obtained from Prolabo (Paris, France).

All solutions were prepared with Milli-Q water (18 MΩ cm, Millipore, Sydney). Buffer solutions used in this work were 50 mM ammonium acetate (pH 7.0) and 0.1 M MES (pH 6.8). The pH was adjusted with either NaOH or HNO_3 solutions. Stock metal solutions (0.1 M) were prepared in Milli-Q water and dilute metal solutions in ammonium acetate. All glassware was rinsed with 6 M HNO_3 followed by Milli-Q water before use to avoid metal contamination.

Preparation of Modified Electrodes

Gold electrodes were prepared by sealing polycrystalline gold wire (>99.99% gold, Aldrich) in 4 mm diameter glass tubes [27]. The cut end of the wire was polished with 1.0 μm alumina, followed by 0.3 and 0.05 μm alumina slurry on microcloth pads (Buehler, Lake Bluff, IL). After removal of the trace alumina from the surface, by rinsing with Milli-Q water and brief cleaning in an ultrasonic bath, the electrodes were further cleaned by electrochemical cycling between -0.3 V and +1.5 V in 50 mM H_2SO_4 at a scan rate of 0.15 V s^{-1} until a reproducible scan was obtained. The electrochemical area of the electrode was determined from the reduction of gold oxide by the method of Hoogvliet *et al.* [28].

Modification of the electrodes with MPA (Figure 1(a)) and TA (Figure 1(b)) were prepared by incubating the electrodes in a 10 mM solution of the corresponding thiol in 75% ethanol, 25% water overnight.

Modification of the electrode with cysteine (Figure 1(c)) was prepared by incubating the electrodes in 10 mM cysteine in ammonium acetate overnight.

Modification of the electrode with MPA-Gly-Gly-His (Figure 1(d)) was prepared by activating the carboxyl terminus of an MPA modified electrodes in a stirred solution of 20 mM EDC and 4 mM NHS in 0.1 M MES (pH 6.8) for 1 hour. After thorough rinsing with MES buffer, the modified electrode was reacted overnight with Gly-Gly-His (20 mg mL^{-1}) in MES buffer to form the MPA-Gly-Gly-His modified electrode.

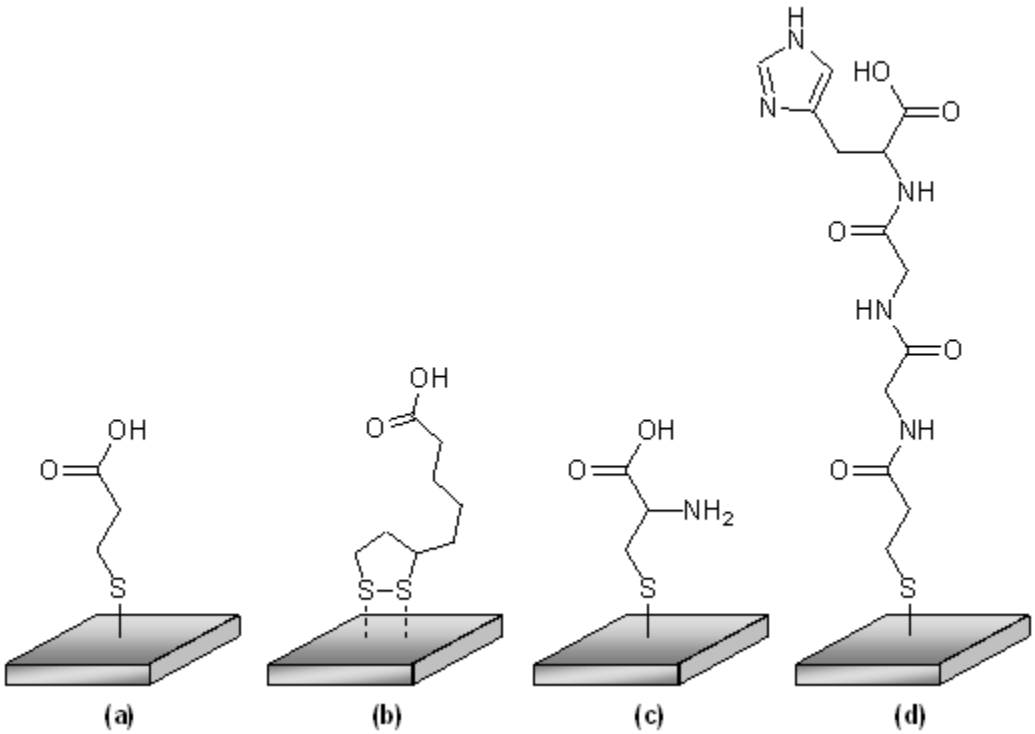

Figure 1. Sensors used for the detection of Cu^{2+}: (a) TA, (b) MPA, (c) cysteine and (d) MPA-Gly-Gly-His modified gold electrodes.

Measurement Procedure

All measurements were made in a water-jacketed cell at 25°C after equilibration of the electrodes in ammonium acetate buffer (pH 7.0) for at least one hour. Copper ions were accumulated at the modified electrode at open circuit potential by immersing the electrode into 10 mL of a stirred aqueous solution of copper(II) sulfate in 50 mM ammonium acetate (pH 7.0) for 10 min. The electrode was removed, rinsed with copper-free ammonium acetate and transferred to a cell with electrolyte of 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl for electrochemical measurements by cyclic voltammetry (CV) and Osteryoung square wave voltammetry (OSWV). After the measurement, bound copper was eliminated from the electrode at +0.5 V for 15 – 30 s in 0.1 M $HClO_4$.

The metal ion concentration of a solution was determined using an ELAN 6100 ICP-MS (Perkin Elmer, Boston, MA) or GBC Integra XMP ICP-AES (Arlington Heights,

IL, USA). All concentrations stated are the measured concentrations except in the interference studies where nominal concentrations of added interferents are stated.

Between four and six electrodes with each modification were fabricated and independently calibrated. The between electrode reproducibility was calculated at each concentration and fitted to a function of the form of Equation (5).

Electrochemical Measurements

All electrochemical measurements were performed with an Autolab PGSTAT 12 potentiostat (Eco Chemie, Netherlands). CV, OSWV and time base experiments were carried out with a conventional three-electrode system, comprising the modified working electrode, a platinum flag auxiliary electrode and a Ag|AgCl|3.0 M NaCl reference electrode (from Bioanalytical System Inc., Lafayette, IN). All potentials are reported versus this reference at 25°C unless otherwise stated. The solution was degassed with argon for approximately 15 minutes prior to data acquisition and was blanketed under an argon atmosphere during the entire experimental period. CV was conducted at a sweep rate of 0.1 V s^{-1} between +0.5 V and -0.3 V for all modified electrodes. In OSWV, the pulse amplitude was 0.025 V with a step of 0.004 V and frequency of 25 Hz. OSW voltammograms were measured between the same potentials for CV experiments. Time base experiments were carried out at +0.5 V for 15 s. In an electrode array, a multiplexer was used to automate the measurements and to switch the measurement from one electrode to another.

Fitting of Calibration Models

Solutions to the algebra required for determining the variance of Equation (2) was achieved using Matlab v6.0, The Mathworks, USA. Fitting of concentration versus current data was performed in Microsoft Excel (Microsoft, Office 2003, Seattle, USA) with the Solver Add-in. The variance-covariance matrix of the fitted parameters was calculated by the Add-in SolverAid [29].

Results and Discussion

Electrochemical Behaviour of Cu^{2+} at Gold Electrodes Modified with TA

The use of TA as the anchor of a modified electrode for metal analysis is described here for the first time. TA also typifies the behavior of the electrodes used in this study and so will be discussed in some detail. The cyclic voltammogram at a TA modified electrode prior to copper accumulation is shown in Figure 2(a)(i). In the absence of any copper, the voltammogram between -0.3 to +0.5 V is electrochemically inactive. After accumulation of the modified electrode in 45 nM copper(II) sulfate, distinct peaks due to the redox

transition Cu^{2+}/Cu^0 appeared with E_a = 0.23 V, E_c = 0.13 V and $E_{1/2}$ = 0.18 V (Figure 2(a)(ii)). In the accumulation process, Cu^{2+} is bound to the carboxyl groups and when the potential is swept in a cathodic direction, Cu^{2+} is reduced to Cu^0 at the electrode surface. Copper is deposited at a potential more positive than the expected Nernstian potential for a bulk process by underpotential deposition (UPD), which has been previously observed to occur through SAMs with less than eight carbon units [30, 31]. Further cycling of the potential at the TA modified electrode resulted in Cu^0 being reoxidised to Cu^{2+} and back again. At a sweep rate of 0.1 V s^{-1}, cycling gave relatively stable peaks indicating little or no loss of copper into the bulk solution. This stability of the peaks could be a consequence of one of two processes. Either all the Cu^{2+} released into solution upon reoxidation of the Cu^0 is captured by TA or the sweep rate during the CV is sufficiently fast that all the released Cu^{2+} is reduced back to Cu^0 at the electrode before it has time to diffuse away. When the sweep rate was slowed to 0.01 V s^{-1} a notable decrease in the peak current was observed with successive scans. This implies that the stability of the electrochemistry at 0.1 V s^{-1} is a consequence of the second possibility; that is the complexed copper is reduced to copper UPD but when reoxidised copper ions are reduced only if they remain close to the surface.

For measurements to quantify the copper concentration, OSWV was used due to the higher sensitivity of this technique. A copper peak current density of 4.3 μA cm^{-2} (s = 0.9 μA cm^{-2}, n = 4 electrodes) was measured at E_c = 0.15 V for the reduction process following accumulation of 45 nM Cu^{2+} for 10 minutes (Figure 2(b)).

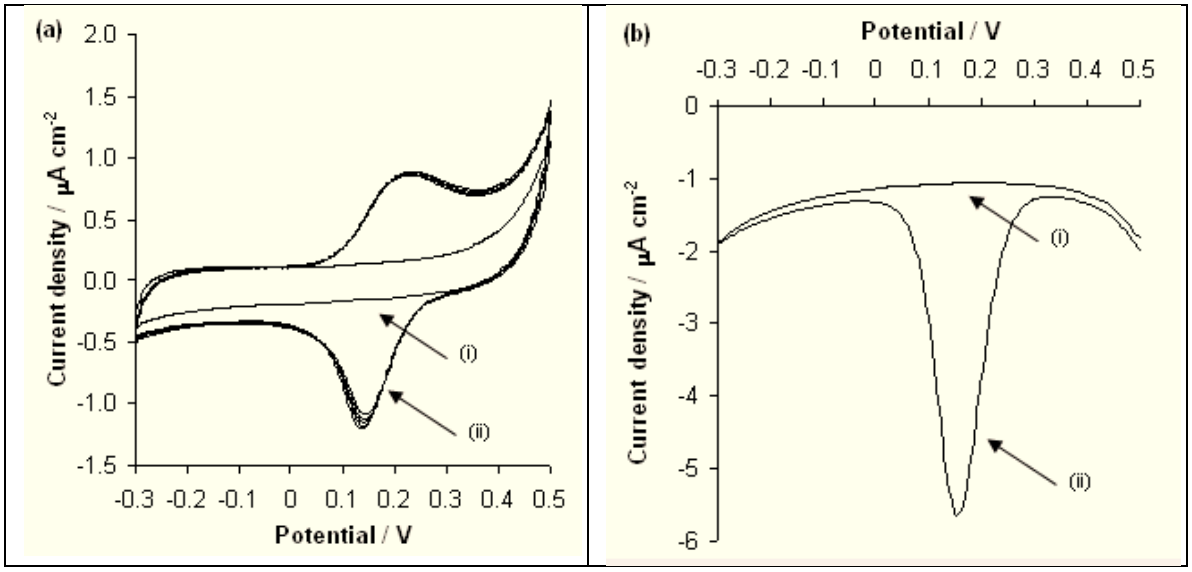

Figure 2. Voltammograms at a TA-modified electrode used for the detection of Cu^{2+} ions. Cu^{2+} ions are complexed to TA in the accumulation process and are electrochemically reduced to Cu^0 to form UPD Cu. (a) Cyclic voltammogram at a TA modified electrode in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl at 25°C at a scan rate of 0.1 V s^{-1} (i) before accumulation of Cu^{2+} and (ii) after accumulation in 45 nM Cu^{2+} in 50 mM ammonium acetate (pH 7.0) for 10 minutes. Multiple cycles in the copper voltammogram illustrate stable electrochemistry. (b) Cathodic OSW voltammograms at a TA-modified gold electrode in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl (i) before accumulation of Cu^{2+} and (ii) after accumulation in 60 nM Cu^{2+} in 50 mM ammonium acetate (pH 7.0) for 10 minutes.

The dependence of the OSWV peak current density at a TA modified electrode on the concentration of Cu^{2+} in the accumulation solution shows two stages in the TA/Cu^{2+} complexation process as illustrated in Figure 3. The lowest Cu^{2+} concentration measured using the TA-modified electrode was 11 nM. Between 25 nM - 0.30 μM, the calibration was approximately linear with sensitivity 30 A cm^{-2} M^{-1} followed by a leveling off between 0.3 – 2.5 μM at 12 μA cm^{-2} signifying all TA sites had taken up Cu^{2+}. The coverage of copper at this point was determined from the charge passed in the cathodic peak of the cyclic voltammogram to be 0.090 nmol cm^{-2} (s = 0.008 nmol cm^{-2}, n = 4 electrodes) assuming a two-electron process. Subsequently, the surface coverage of TA was determined by reductive desorption from -0.3 V to -1.4 V in 0.5 M KOH, at a scan rate of 0.1 V s^{-1}. The coverage of TA was 0.50 nmol cm^{-2} (s = 0.07 nmol cm^{-2}, n = 4 electrodes) was obtained from which the ratio of copper to TA coverage was calculated to be 1:5.6 ± 2.3 (95% confidence interval). This value includes the expected ratio of 1:4 for the formation of a tetradentate complex. At concentrations above 2.5 μM, the current begins to rise again due to the formation of a more copper rich complex with TA. Between 2.5 μM – 40 μM the sensitivity is 1.0 A cm^{-2} M^{-1} with a plateau at concentrations around 1.0 mM and current density of 112 μA cm^{-2}. Concentrations above 1.0 mM Cu^{2+} (prepared in 50 mM ammonium acetate buffer (pH 7.0)) resulted in the formation of a precipitate and were not used for further calibration. The coverage of copper at this concentration was 0.22 nmol cm^{-2} (s = 0.02 nmol cm^{-2}, n = 4 electrodes) which leads to a ratio of 2.3 ± 0.9 (95% confidence interval). This ratio implies a 1:2 complex, $Cu(RCOO)_2$.

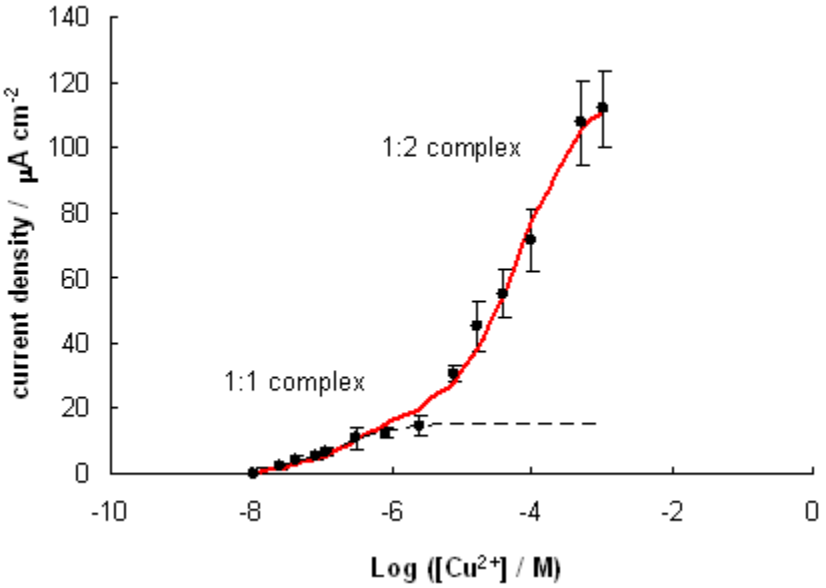

Figure 3. OSWV peak current density for the reduction of Cu^{2+} at a TA modified gold electrode as a function of log(Cu^{2+}) concentration. Error bars are ±1 standard deviation of the current densities of four individual electrodes. Experimental conditions as given for the caption in Figure 2(b). Solid line is a fit to Equation (2) with $I_{0,1}$ = 18.3 (3.2) μA cm^{-2}, $K_{M,1}$ = 3.9×10^6 (2.1×10^6) mol^{-1} L, $I_{0,1}$ = 99.1 (3.7)

μA cm^{-2}, $K_{M,1}$ = 1.5×10^4 (0.2×10^4) mol^{-1} L. Dashed line is fit of first part of data to a single function, which models the 1:1 complex. Figures in parentheses are the standard errors of the parameters.

The standard deviations of the currents from replicate electrodes followed a function

$$s = 0.44 + 7.30 \times \log_{10}[M] + 30.9 \times (\log_{10}[M])^2 \quad (8)$$

(see Figure 4). This provides a reasonable and continuous function to use in calculation of uncertainties of measurements of concentrations. The reproducibility of the current from different electrodes falls from over 50% at 10^{-8} M to about 10% at the highest concentrations. A large within electrode repeatability was observed, although the means of currents were remarkably constant across electrodes.

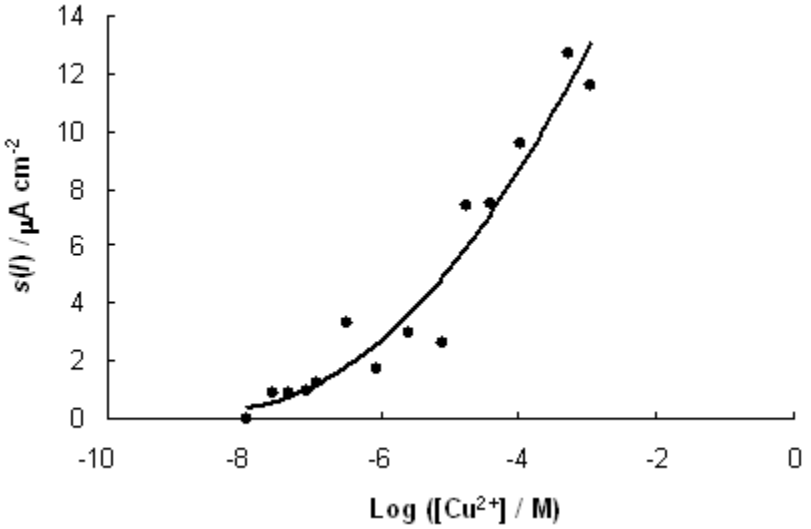

Figure 4. Standard deviation of the currents from replicate electrodes modified with TA as a function of log(Cu^{2+}) concentration. Solid line is a fit to Equation (11).

The variance due to the fit was calculated from Equation (7) and is illustrated in Figure 5. The increase in uncertainty in the middle of the range coincides with the saturation of the first species, the 1:1 complex. As the sensitivity rises when the 1:2 complex is formed the uncertainty falls until the end of the range when again saturation of the complex leads to low sensitivity. Although the RSD's are in the tens of percent, this is remarkably good for the reproducibility of independently prepared electrodes, and implies the possibility of mass production of electrodes to a constant performance.

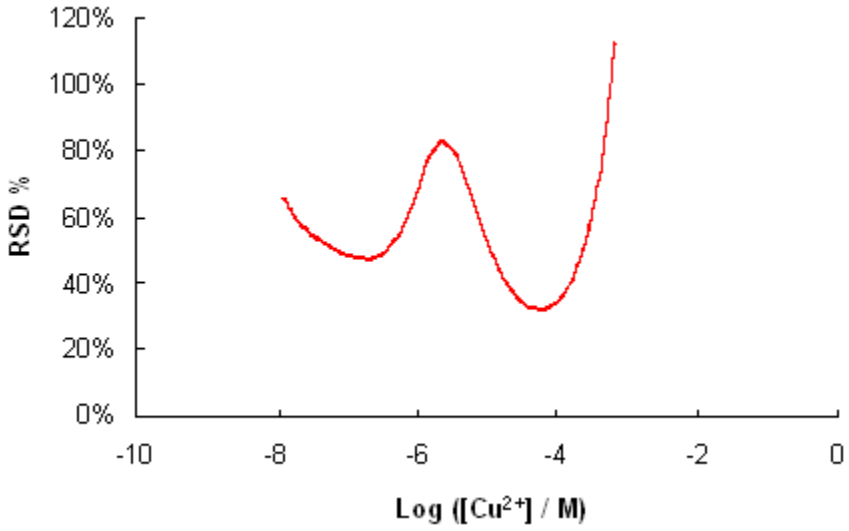

Figure 5. Relative standard deviation (RSD) of the concentration of copper estimated from the fit of Figure 3, as a function of log copper concentration.

One desirable characteristic of a sensor is high selectivity i.e. the ability to bind the target metal with minimal interference from potentially interfering ions. The selectivity of the TA-modified electrodes for Cu^{2+} in the presence of Zn^{2+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Ba^{2+} and Cr^{3+} was investigated using a two-level Plackett-Burman experimental design [32] as shown in Table 1. The main effect of a factor is the average change in response variable (here current density) as the factor is changed from its low value (designated by the contrast coefficient -1) to its high value (contrast coefficient +1). Each solution contained a nominal 200 nM Cu^{2+} plus either a low concentration (200 nM, -1 level) or high concentration (5 μM, +1 level) of each interferent. A 7-variable, 8-experiment design was used with six interferent factors and a dummy factor. The dummy factor (one which clearly has no effect) allows an estimate of the measurement variance. To determine the effect of a factor, the responses multiplied by their contrast coefficients are summed, and then divided by half the number of runs. The significance of an effect was then inferred from a Rankit plot (or normal distribution plot) which is constructed by plotting the expected normal z-score of the rank of a particular effect against the value of the effect. If the data have only random errors that are normally distributed then the effects should lie on a straight line close to zero. Significant effects fall off the line. As illustrated in Figure 6, Ni^{2+}, Ba^{2+}, Cd^{2+}, Zn^{2+}, Cr^{3+} and the dummy variable may all be considered insignificant. The only significant effect is Pb^{2+} which leads to a decrease in the copper current density of -1.3 μA cm^{-2} or 30% of the mean current density with no interferents (4.3 μA cm^{-2}). Pb^{2+} being an interferent is not surprising considering it is a borderline metal and has no preference for hard/soft donor atoms and can easily interact with copper binding sites. However, even though Cr^{3+} is expected to interfere, since it is classified as a hard metal and prefers oxygen donors, Cr^{3+} only showed a minor negative effect of -0.4 μA cm^{-2} (-9%) which was deemed statistically insignificant.

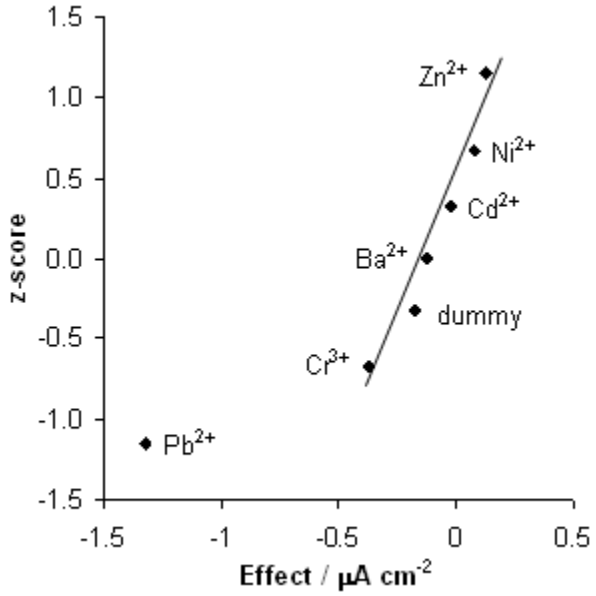

Figure 6. Rankit plot for the cathodic OSWV copper peak current density effects of interferents at a TA modified gold electrode. Experimental conditions as given for Table 1.

Table 1. Contrast coefficients for a Plackett-Burman experimental design for the interference studies of copper complexation to the modified electrodes. +1 represents high concentration (5 μM) and -1 represents low concentration (200 nM) of the interfering ion. The experiments were conducted with a constant concentration of 200 nM Cu^{2+}.

Run	Zn^{2+}	Pb^{2+}	Ni^{2+}	Cd^{2+}	Ba^{2+}	Cr^{3+}	Dummy
1	-1	-1	-1	+1	+1	+1	-1
2	+1	-1	-1	-1	-1	+1	+1
3	-1	+1	-1	-1	+1	-1	+1
4	+1	+1	-1	+1	-1	-1	-1
5	-1	-1	+1	+1	-1	-1	+1
6	+1	-1	+1	-1	+1	-1	-1
7	-1	+1	+1	-1	-1	+1	-1
8	+1	+1	+1	+1	+1	+1	+1

Electrochemistry of Cu^{2+} at Gold Electrodes Modified with MPA, Cysteine and MPA-Gly-Gly-His

Although the TA modified electrodes exhibit a wide dynamic range for copper (between 11 nM - 0.3 μM and 2.5 μM - 1.0 mM), the sensor is best used in the second stage of the calibration where it has higher sensitivity. MPA, MPA-Gly-Gly-His and cysteine modified electrodes were also evaluated and their performance parameters are listed in Table 2.

Table 2. Performance parameters of electrochemical Cu^{2+} sensors based on SAM-modified electrodes.

Modified Electrode	Lowest detectable concentration	Dynamic range (pK_{Cu})	Maximum current density	Significantly interfering Ions
TA	11 nM	8.1 - 3.0	112 μA cm^{-2}	Pb^{2+}
MPA	1.6 nM	8.8 - 3.0	211 μA cm^{-2}	Pb^{2+}, Cr^{3+} and Cd^{2+}
Cysteine	3.1 nM	8.5 - 6.1	36.5 μA cm^{-2}	Pb^{2+}, Cr^{3+} and Ba^{2+}
MPA-Gly-Gly-His	0.3 nM	9.0 - 4.5	158 μA cm^{-2}	Pb^{2+} and Cr^{3+}

The MPA modified electrodes behave similarly to TA modified electrodes in the presence of Cu^{2+} since they both possess the carboxyl moiety, but with higher Cu^{2+} currents measured at the MPA modified electrode. In comparison, the analytical performance of the cysteine sensor is inferior to MPA but superior to TA with regards to sensitivity. It has been reported that cysteine modified electrodes bind to copper through the carboxyl and amino groups to form a 1:2 copper:cysteine complex [7]. However, at the pH of the solution used for Cu^{2+} accumulation (7) the ammonium ions are mostly protonated and therefore no lone pairs are available for copper complexation. Binding of Cu^{2+} to Gly-Gly-His results in the formation of a highly stable 4N square planar complex [33, 34]. The sensor displayed a wide dynamic range, with concentrations ranging from 0.3 nM and as high as 1 mM able to be determined. The lowest measurable concentration of 0.3 nM demonstrates the superb copper-binding ability of electrodes modified with MPA-Gly-Gly-His (see Table 2).

Comparisons Between the Four Kinds of Sensors

As illustrated with the four kinds of sensors, there are several factors which determine the copper peak current density. First, the surface coverage of the recognition molecule has implications for the number of sites available for complexation. A greater surface coverage means more binding sites available to the metal ion. For MPA, an average surface coverage of 0.74 nmol cm^{-2} was determined which is a greater value than any of the other electrode interfaces. This interface resulted in high copper currents. With the cysteine modified gold electrodes, the surface coverage of the SAM was 0.61 nmol cm^{-2} although only a small fraction of the sites were available as COO^- and NH_2 for copper complexation resulting in lower currents. For TA, which has a surface pK_a of 2.75 [35], it should be deprotonated under neutral conditions and hence be able to complex copper species effectively. However, the affinity of the metal ion for the ligand is another important consideration. The most effective ligand for Cu^{2+} is Gly-Gly-His as this results in the formation of a highly stable 4N square planar complex. Hence the Gly-Gly-His modified electrode exhibited the lowest detection limit for Cu^{2+} (0.3 nM) over the other sensors used in this study. Whether Cu^{2+} forms a 1:1 complex with the ligand or 1:2 or 1:4 also has an effect as this will determine the maximum amount of Cu^{2+} that is able to bind to the surface. Finally, there is the distance between the redox active centre and the electrode. Cu^{2+}, which is held further away from the electrode when the surface is modified with TA, will produce lower currents than a surface modified with MPA. From measurements of the separation of the anodic and cathodic peaks in cyclic voltammetry as a function of sweep rate, the electron transfer rate constant was calculated, by the Laviron equation [36], to be k_{ET} = 10.4 s^{-1} (s = 3.0 s^{-1} , n = 4 electrodes) for an MPA electrode and 3.3 s^{-1} (s = 0.4 s^{-1}, n = 4 electrodes) for a TA modified electrode. The difference in rate constants is commensurate with the carbon chain lengths.

Combining these sensors into an array has several advantages for detecting Cu^{2+} (see Figure 7). Although the MPA-Gly-Gly-His modified electrodes (Figure 7(ii)) displays a very wide dynamic range, it is most useful for detecting very low concentrations of Cu^{2+}. At these low levels, the other sensors are not expected to respond and they can be useful in verifying that the concentration of Cu^{2+} in a sample solution is in fact very low. At higher concentrations, all sensors will respond to Cu^{2+} with the MPA-Gly-Gly-His and MPA modified electrodes (Figure 7(i)) displaying the highest copper peak current densities. However, the current density of the MPA-Gly-Gly-His sensor only rises slowly between micromolar to millimolar concentrations of Cu^{2+}. For efficient detection of high concentrations of Cu^{2+}, TA (Figure 7(iv)) is perhaps the best sensor with slightly higher sensitivity over the MPA sensor and much better sensitivity than MPA-Gly-Gly-His. The practical working range of the TA sensor is between 2.5 μM - 40 μM Cu^{2+} with a copper sensitivity of 1.0 A cm^{-2} M^{-1}. The TA modified electrode also displays better copper selectivity than the MPA modified electrode with only Pb^{2+} identified as a significantly interfering ion. So for detecting Cu^{2+} at a wide dynamic range using the sensor array approach, there are two sensors which must be incorporated, MPA-Gly-Gly-His and TA. However, there is still potential in combining all four electrodes into a sensor array, with the MPA and cysteine modified electrodes (Figure 7(iii)) playing contributory roles [37]. Since none of the modified electrodes are entirely specific to copper, the current will be affected by the presence of interfering ions to some extent. If

all electrodes are exposed to the same sample solution, the same concentration of copper should be predicted from each of the calibration plots in the absence of interferences. In the presence of interfering ions, a smaller copper current will be obtained which will consequently predict a smaller concentration. In this situation, the predicted concentration using the four different electrodes will not all necessarily be the same.

In a sensor array approach, the problem of interfering ions may be taken into account as each modified electrode is affected to a different extent. If the interfering ions are electrochemically active, then they can be quantified by multivariate techniques even when the voltammograms are extensively overlapped [38].

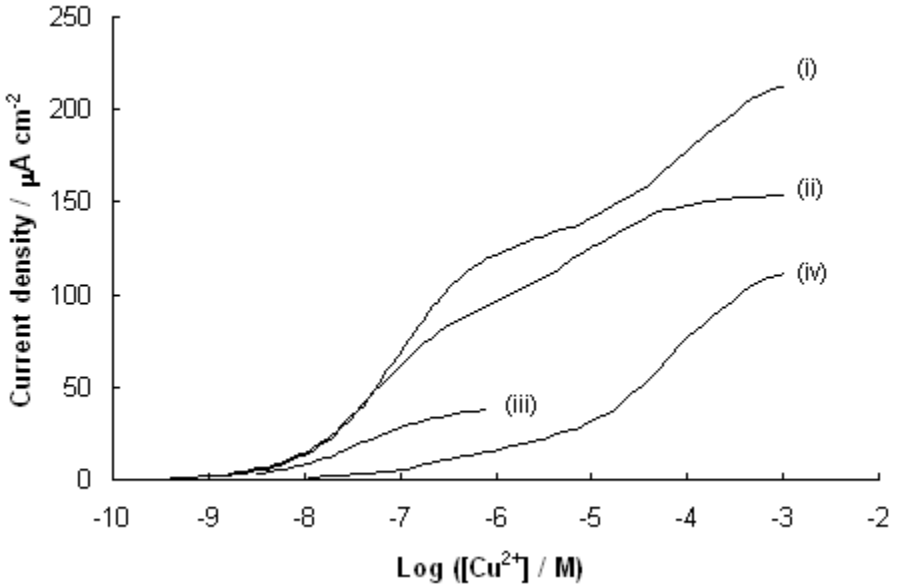

Figure 7. Calibration curves for the four modified electrodes: (i) MPA, (ii) MPA-Gly-Gly-His, (iii) cysteine, (iv) TA. Current densities were determined from the peak OSW voltammograms.

Combining Data from Different Electrodes

Calibration curves for each electrode are shown in Figure 7. The method of combining the results advocated here is to calculate the concentration of copper for each electrode in the range of its calibration, and weight the contribution by the variance of the measurement. This does not lose information, as calibration of the summed currents would, nor does it have the complexity of a multivariate calculation, especially with electrodes having different calibration ranges.

The uncertainty from the calibration fits and reproducibility of the current measurement for each electrode can also be combined as a pooled variance. The best estimate of the concentration $[M]_{pooled}$ is given by:

$$[M]_{\text{pooled}} = \frac{\sum_{i=1}^{i=4} \frac{[M]_i}{V_{i,\text{M}}}}{\sum_{i=1}^{i=4} \frac{1}{V_{i,\text{M}}}} \qquad (9)$$

where the sum is over the four electrodes, $[\text{M}]_i$ is the calibrated result of electrode i and $V_{i,\text{M}}$ the variance of electrode i at concentration $[\text{M}]_\text{i}$.

The pooled variance is

$$V_{\text{pooled}} = \frac{1}{\sum_{i=1}^{i=4} \frac{1}{V_{i,\text{M}}}} \qquad (10)$$

The greater the variance (uncertainty) the smaller the contribution of the result from an electrode to the pooled value. Outside the calibrated range the variance is set at infinity (i.e. $1/V = 0$) and so the electrode contributes neither to the pooled concentration nor the variance. Figure 8 gives the RSD for each electrode and the combined RSD across the range of concentrations of the four electrodes.

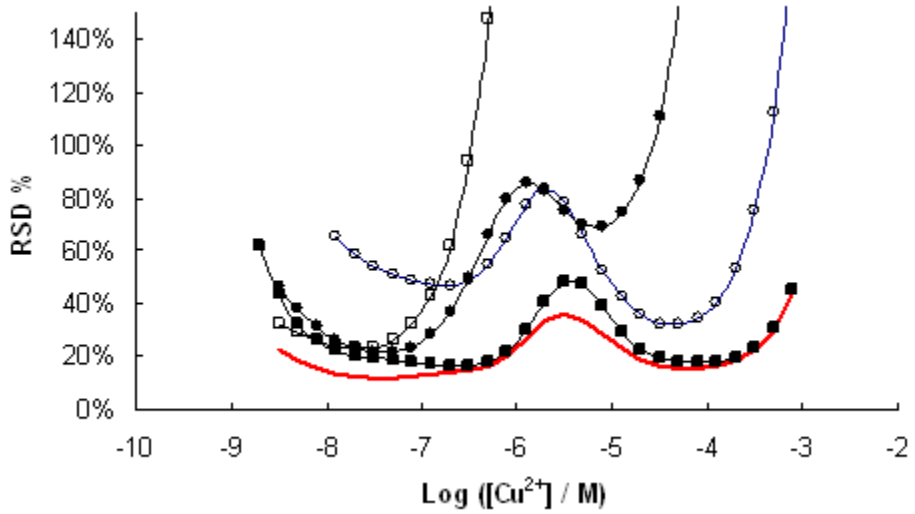

Figure 8. RSD of the estimated concentrations of four electrodes: TA (open circles), MPA (closed squares), cysteine (open squares) and MPA-Gly-Gly-His (closed circles), solid line: pooled uncertainty.

The fact that the standard deviation used in the estimate of measurement uncertainty is from a number of independently-prepared electrodes implies that electrodes could be produced in batches and factory calibrated with acceptable precision. When used in the field the electrode could be checked with a quality control solution, but would not require further calibration.

Conclusions

This paper has demonstrated how to use a non-linear calibration function across its full range, by tracking the uncertainty of the estimated concentration. Also described is how to combine a number of calibration functions that only partially overlap to give a better (i.e. with smaller variance) estimate of the concentration of the analyte. The accumulation and voltammetric reduction of copper ions at four gold electrodes, modified with different complexing species (3-mercaptopropionic acid, thioctic acid, and the peptides cysteine and Gly-Gly-His) was used as an illustrative system. As the equilibrium complexation of a target analyte represents a common motif in many sensors, for example in enzyme and immuno-sensor binding, the methods reported here should find use more widely.

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