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Electrochemical detection of lead ions via the covalent attachment of human angiotensin I to mercaptopropionic acid and thioctic acid self-assembled monolayers

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Abstract

An electrochemical sensor for the detection of lead ions is described which is made by modifying a gold electrode substrate with self-assembled monolayers (SAMs) of 3-mercaptopropionic acid (MPA) or thioctic acid (TA) followed by covalent attachment of a lead binding peptide, human angiotensin I. Cyclic voltammetry of MPA–angiotensin modified gold electrodes complexed with lead displayed voltammograms with prominent lead peaks at $E^{0'}$, −0.29 V. A detection limit of 1 nM was achieved using Osteryoung square wave voltammetry. However, the electrodes were not stable over repeated electrochemical cycles due to partial electrochemical desorption of the SAM. The TA–angiotensin modified gold electrode showed greater stability and were able to be regenerated several times. Using Osteryoung square wave voltammetry for TA–angiotensin modified electrodes, lead concentrations down to 1.9 nM were detected. Although the detection limit of the TA–angiotensin modified electrode is higher than achieved with MPA–angiotensin, it is still well below Australian drinking water guidelines. Studies of interference effects on the Pb^{2+} current showed Hg^{2+} as a significant interferent but only at levels significantly greater than those found in natural waters.

Keywords: Biosensors; Electrochemistry; Lead; Angiotensin; Heavy metals

1. Introduction

Lead is an environmental contaminant with exposure to humans arising mostly from contaminated soils and accumulation from food. The greatest risk is to children who can retain over 30% of ingested lead whereas for adults less than 5% is retained [1]. The Australian drinking water guideline for lead is 48 nM (10 ppb) [2]. The effects of Pb^{2+} toxicity can be severe including renal malfunction and nervous system disorders plus inhibitory developments in fetal and child brains [1]. In the past few decades, there has been an increased awareness of the effects of lead toxicity with a phasing out of lead additives to paints and fuels. Its toxicity arises owing to its borderline (hard–soft) character. It can bind at sites of many biomolecules, which can cause alteration or loss of biological function. Lead ions can interfere directly with calcium signaling, since it has a similar ionic radius to Ca^{2+} and hence the ability to substitute for calcium [1]. It is also known to inhibit several zinc enzymes [1,3–6], for example, substituting for zinc in 5-aminolevulinic acid dehydratase (ALAD), which is important in the biosynthesis of heme [6].

The development of biosensors as a method of analysis for low concentrations of metal ions is a relatively new and promising area [7–12]. Biosensors are ideal devices for analyses in the field having the desirable characteristics of simple use and cheap manufacture. They are superior to chemical sensors for metal ion detection since the recognition molecule is a biological molecule and hence could provide information on metal ion interactions with a particular organism. Since the toxicity of a metal is related to the amount of bioavailable metal and not total metal content [11], recognition elements which incorporate enzymes and proteins of organisms are suitable systems to study. A fluorosensor for

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Pb^{2+} was developed by Li and Lu [8] with a detection limit of 10 nM and >80-fold selectivity for Pb^{2+} over other divalent metal ions. In this system, Li and Lu used a Pb^{2+}-specific ribozyme, which was hybridized with the complementary sequence of DNA which was labeled with a fluorophore at the 5′ end. Hybridization brought the fluorophore next to a quencher attached to the ribozyme. Upon addition of lead, a link in the DNA was cleaved by the ribozyme, resulting in denaturation of the duplex and removal of the fluorophore from the quencher to give a 400 times increase in fluorescence. In a different transduction approach, an electrochemical enzyme biosensor for the detection of Pb^{2+} was developed by Veselova and Shekhovtsova [10]. This sensor relied on Pb^{2+} inhibiting the activity of alkaline phosphatase immobilized in *N*-phthalylchitosan on polyurethane foam. The detection limit for Pb^{2+} was 0.10 nM. The key drawback of the inhibition-based enzyme biosensor is selectivity, which showed that Bi^{3+}, Cd^{2+} and Zn^{2+} influenced the determination of 0.24 nM Pb^{2+}. Kremleva et al. [9] have also developed an electrochemical method for determining lead and cadmium using an amperometric cysteine desulfhydrolase tissue biosensor. The response to the action of the metal ions was judged from a change in the catalytic activity of cysteine desulfhydrolase. Detection limits for cadmium and lead were 10 and 30 nM, respectively, for this system.

Self-assembled monolayers (SAMs) of thiols on gold electrodes are an attractive means by which a biological recognition element can be anchored onto the surface of an electrode [13–16]. SAMs can be tailored with various functionalities, which enable further modification with recognition molecules. Our research group has used this concept to develop electrochemical metal ion biosensors in which peptides are anchored to carboxylic acid terminated thiols [17–23]. This approach involves identifying peptide sequences, which mimic the natural binding sites of organisms in order to potentially provide information about bioavailability of the metal in question. Once the peptide is attached, the binding of metal ions may be transduced by exploiting the electrochemistry of the metal, or changes in fluorescence of the system. Our group has concentrated on observing the voltammetry of the bound metal ion.

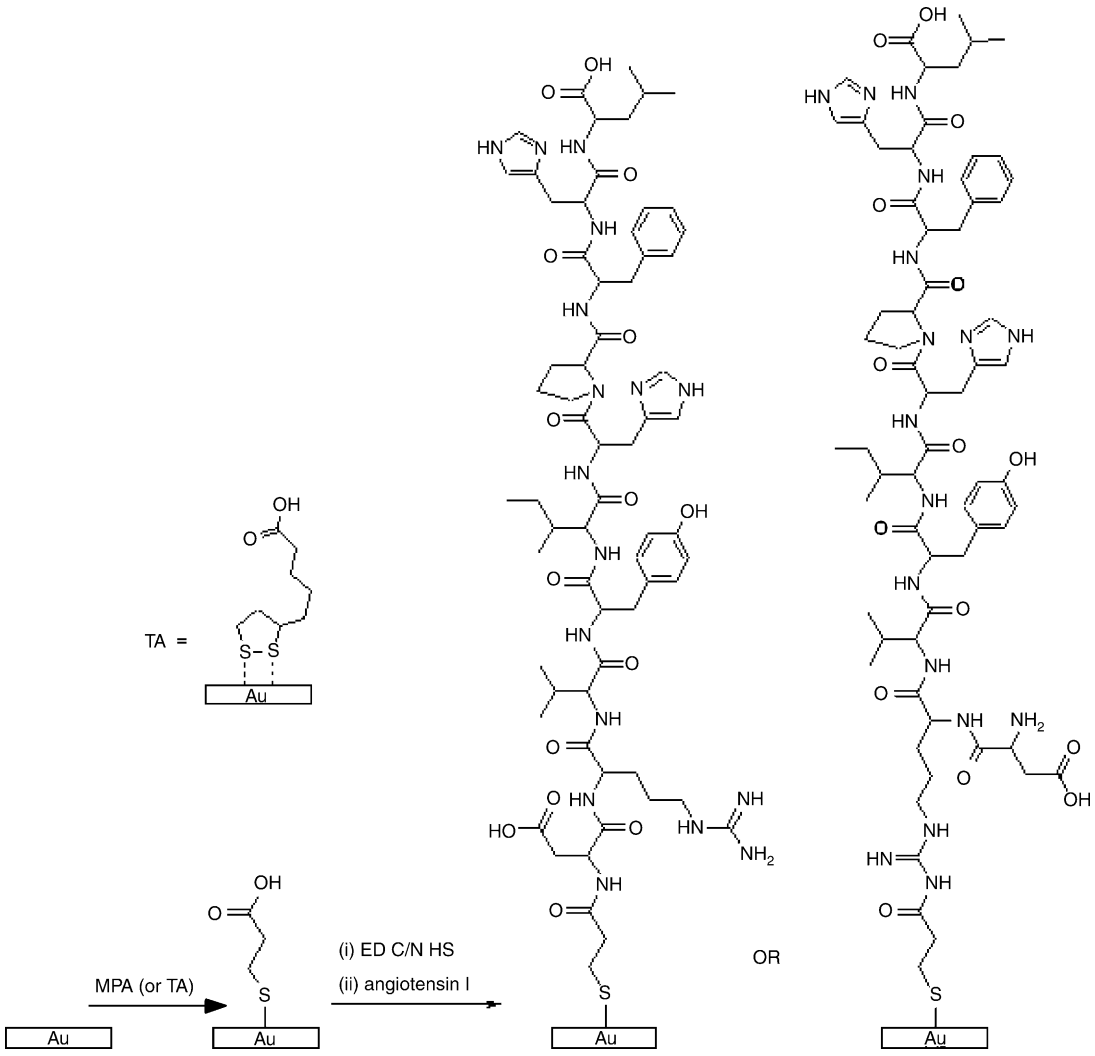
In our earlier work, we chose 3-mercaptopropionic acid (MPA) as the carboxylate linker to the peptide. Using carbodiimide coupling, both Gly–Gly–His [18–21] and polyaspartic acid modified electrodes [17] for detecting copper ions and γ-Glu–Cys–Gly (GSH) [22] and His–Ser–Gln–Lys–Val–Phe (HSQKVF) [23] modified electrodes for detecting cadmium ions were developed [22,23]. SAMs of MPA are the most exploited [14,17–23,25,26] since the short alkyl chain allows anchoring of the recognition species without passivation of the underlying electrode. Short chain SAMs, however, have only a limited potential range (typically −0.6 to +0.6 V) in which they are stable so it is important that the electroactive species falls within this range [27]. There are however numerous other carboxyl-terminated thiols which are commercially available of differing chain length and hydrophobicity. Longer chain mercaptoalkanoic acids, for example, 11-mercaptoundecanoic acid, MUA, are stabilized by intermolecular van der Waals forces. However, the disadvantage of long chain thiols for electrochemistry is that the site of redox activity will be remote from the electrode, giving a low rate of electron transfer and hence any current will be small [28]. A compromise between sensitivity and stability is thioctic acid (TA), which has two thiol groups which chemisorb to the electrode to provide greater enhanced stability without removing the recognition molecule being located at a significantly greater distance from the electrode than MPA [25,29–33].

In identifying a peptide for lead, an obvious choice is a zinc protein, which Pb^{2+} inhibits. There are many zinc fingers with motifs of the type Cys–Cys–His–His, Cys–Cys–His–Cys or Cys–Cys–Cys–Cys which Pb^{2+} is able to bind more tightly to than Zn^{2+} [1,3–5,34]. However, these sequences are normally greater than 30 amino acids long which can be problematic for electrochemical interrogation of a metal immersed in such a large peptide. Another peptide of shorter length which binds to Zn^{2+} is human angiotensin I which has the amino acid sequence Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu. Angiotensin I has histidine residues at positions 6 and 9 that are involved in metal coordination as demonstrated by Loo and co-workers [35,36]. It was also suggested by these researchers that metal ions are only coordinated to one of the histidines and to the two immediate carbonyl groups, imparting minimal constraints on the peptide. Bidentate coordination by the two histidine residues is also possible. Using the analogy that Pb^{2+} commonly binds more strongly to zinc fingers than zinc itself we hypothesize that angiotensin I may have a high affinity for Pb^{2+} as well and perhaps a higher affinity for Pb^{2+} than Zn^{2+}. The purpose of this paper is to report on the performance of MPA–angiotensin I and TA–angiotensin I modified gold electrodes for the detection of Pb^{2+}. The peptide is attached to carboxylic acid terminated SAMs using a combination of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC and *N*-hydroxysuccinimide (NHS) which converts the carboxylic acid to a succinimide ester which is susceptible to nucleophilic attack from amines on the peptide to form an amide bond (see Scheme 1). In the case of angiotensin, the attachment can occur via the amino group of Asp or Arg as well as the N-terminus of the peptide. However, since neither of these residues are involved in Pb^{2+} complexation, there was no preference for which residue attached to the carboxylate and hence no protecting groups were necessary for either of these residues in the coupling step (Scheme 1).

2. Experimental

2.1. Materials

Human angiotensin I, D,L-6,8-thioctic acid and *N*-hydroxysuccinimide (NHS) were purchased from Sigma (Sydney, Australia). 3-Mercaptopropionic acid (MPA),



Scheme 1. General experimental conditions for the fabrication of MPA–angiotensin I and TA–angiotensin I modified gold electrodes. Electrodes are modified in 10 mM MPA/TA solution overnight followed by activation of the carboxyl groups using EDC/NHS to allow for the attachment of angiotensin I. Angiotensin I can attach to MPA/TA through the amino end of the Arg or Asp residue.

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(*N*-morpholino)-ethanesulfonic acid (MES), barium(II) nitrate, zinc(II) nitrate, mercury(II) oxide, silver(I) nitrate and $HClO_4$ were from Aldrich (Sydney, Australia). Sodium 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 (particularly Cu^{2+}) contamination. The metal ion concentration of the calibrator and sample solutions were determined using an ELAN 6100 ICP-MS (from Perkin-Elmer, Boston, MA). All concentrations stated are the concentrations measured by ICP-MS except in the interference studies where nominal concentrations of added interferents are stated.

2.2. Preparation of MPA and MPA–angiotensin I modified gold electrodes

Gold electrodes were prepared by sealing polycrystalline gold wire (>99.99% gold, Aldrich) in 4 mm diameter glass tubes with EPON Resin 825 and EPI-CURE curing agent 3271 from Shell (Sydney, Australia) [37]. 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 trace alumina from the surface, by rinsing with Milli-Q water and sonicating for 5 min, the

electrodes were further cleaned by electrochemical cycling between −0.3 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. using a conversion factor of 482 μC cm^{-2} [38].

Modification of the gold electrode with MPA–angiotensin I was performed as outlined in Scheme 1. First, the electrode was incubated overnight in a 10 mM solution of MPA in 75% ethanol, 25% water followed by rinsing with absolute ethanol. The carboxyl terminus was then activated by immersing the MPA modified electrode in a stirred solution of 20 mM EDC and 4 mM NHS in 0.1 M MES (pH 6.8) for 1 h. After thorough rinsing with MES buffer, the modified electrode was reacted overnight with angiotensin (10 mg mL^{-1}) in MES buffer at 4 °C to form the MPA–angiotensin I modified gold electrode.

2.3. Preparation of TA and TA–angiotensin I modified gold electrodes

The preparation of TA and TA–angiotensin I modified gold electrodes were as described for MPA and MPA–angiotensin I, respectively, substituting TA for MPA (Scheme 1).

2.4. Measurement procedure

All measurements were made in a water-jacketed cell at 25 °C after equilibration of the electrodes in ammonium acetate buffer for at least 1 h. Lead ions were accumulated at the modified electrode at open circuit potential by immersing the electrode into 10 mL of a stirred aqueous solution of lead(II) nitrate in 50 mM ammonium acetate (pH 7.0) for 10 min. The electrode was removed, rinsed with lead-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 lead was eliminated from the electrode at +0.5 V for 10 s in 0.1 M $HClO_4$.

2.5. 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 a bare or modified working electrode, a platinum flag auxiliary electrode and a Ag |AgCl| 3.0 M NaCl reference electrode (from Bioanalytical Systems 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 min prior to data acquisition and was blanketed under an argon atmosphere during the entire experiment. Cyclic voltammetry was performed at a sweep rate of 0.1 V s^{-1} between +0.2 and −0.44 V for MPA and MPA–angiotensin modified electrodes and between +0.2 and −0.4 V for TA and TA–angiotensin 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 as for CV experiments. Time base experiments were carried out at +0.5 V for 15 s.

3. Results and discussion

3.1. Detection of Pb^{2+} using MPA–angiotensin I modified gold electrodes

Shown in Fig. 1(a) is the cyclic voltammogram of an MPA–angiotensin I modified electrode (i) before and (ii) after accumulation in 50 nM Pb^{2+} cycled between +0.2 and −0.44 V in 50 mM ammonium acetate buffer (pH 7.0) and 50 mM NaCl. The observed redox chemistry in Fig. 1(a) (ii) at E_c −0.37 V and E_a −0.21 V with $E^{0'}$ −0.29 V is due to the Pb^{2+}/Pb couple. In the accumulation process, Pb^{2+} is bound to angiotensin, whereas upon electrochemical reduction Pb is underpotentially deposited (UPD) onto the gold surface beneath the sulfur atom. UPD is the process in which a metal deposits on a foreign substrate at a potential more positive than the reversible Nernst potential for bulk metal formation [39]. In the stripping of deposited Pb, a broad oxidation peak resulted. The fact that the peak appeared broad rather than sharp is indicative that the process is not a true stripping peak where the lead is removed from the vicinity of the electrode. It is postulated that Pb^{2+} is recaptured by angiotensin when it is reoxidised, thus leading to a broad signal. The standard electrode potential for Pb^{2+}/Pb is −0.35 V whereas UPD of lead has been observed at a bare gold electrode at approximately −0.2 V under acidic conditions [40]. The UPD potential shifted negatively by coating the electrode with a SAM which is in agreement with the results of Yoneyama and co-workers [40]. UPD on gold has also been reported for Cu [40–44], Cd [40,45] and Ag [40,46]. Yoneyama and co-workers [47] have shown that Cu can UPD through a SAM of propanethiol and stripping of Cu can take place reversibly without any loss of the SAM. Reductive desorption experiments also provide evidence that the SAM is stabilized by the Cu monolayer [47]. The baseline in the CV of the MPA–angiotensin modified electrode after Pb^{2+} accumulation shifted to higher currents compared to the blank which is indicative of a change in the properties of the SAM. For five successive cycles of the MPA–angiotensin/Pb^{2+} modified electrode, the peak current decreased which implies that Pb^{2+} is not recaptured efficiently by the angiotensin such that the Pb^{2+} is lost into the bulk solution over time. Alternatively, the decrease in signal could be attributed to the MPA–angiotensin/Pb^{2+} complex being reductively desorbed from the electrode surface at the extremes of the cathodic potential used in the electrochemical cycling. These will be discussed later in this paper.

For analytical measurements, rather than use CV, OSWV (Fig. 1(b)) was performed due to the higher sensitivity of this

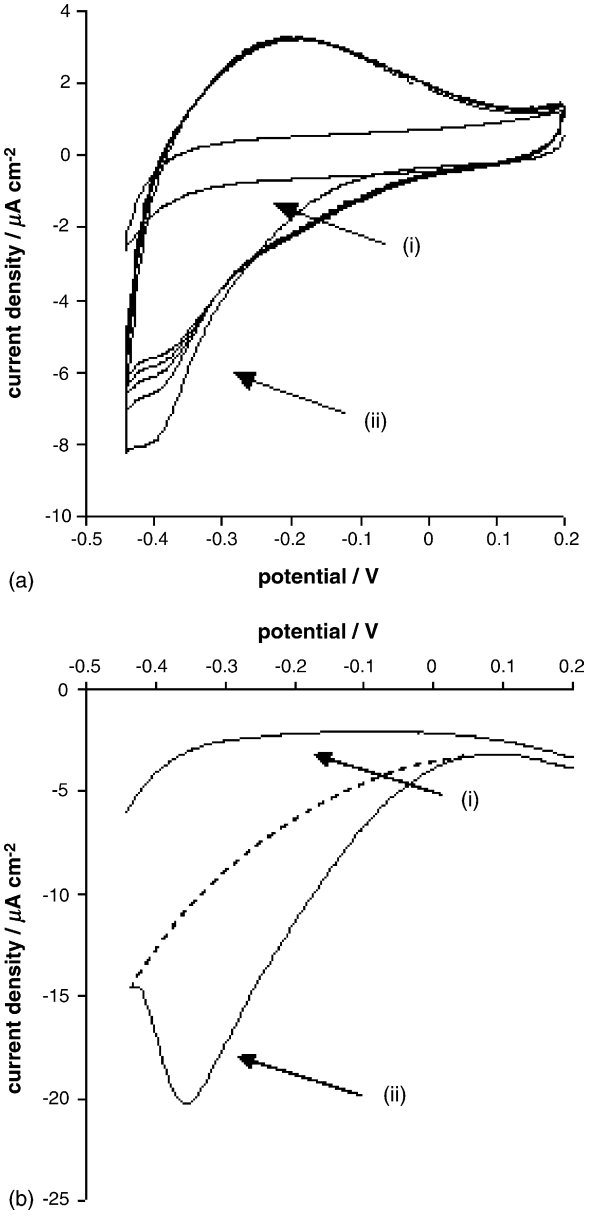


Fig. 1. (a) Cyclic voltammograms of an MPA–angiotensin I modified gold electrode in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl (i) before accumulation of metal ions and (ii) after accumulation in 50 nM Pb^{2+} in 50 mM ammonium acetate (pH 7.0) for 10 min. Multiple cycles in the voltammogram illustrate decreasing current in the reduction wave. Scan rate: 0.1 V s^{-1}. (b) Cathodic Osteryoung square wave voltammograms of an MPA–angiotensin I modified gold electrode in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl (i) before accumulation of metal ions and (ii) after accumulation in 50 nM Pb^{2+} in 50 mM ammonium acetate (pH 7.0) for 10 min.

electrochemical technique as a consequence of double layer charging not contributing to the background signal. The peak current density for the reduction of Pb^{2+} was 7.6 μA cm^{-2} (s = 1.1 μA cm^{-2}, n = 4) at −0.36 V. Thus, OSWV was used for all further quantitative measurements.

For the purpose of developing a sensor, one potentially important criterion is the reusability (i.e., the stability of the electrode). The regeneration of MPA–angiotensin/Pb^{2+} modified electrodes was investigated by measuring the peak cathodic current density in the OSW voltammogram with each accumulation in 50 nM Pb^{2+}. Removal of lead from the electrode was achieved by holding the potential at +0.5 V in 0.1 M $HClO_4$ for 15 s. Subsequent accumulation/removal cycles of the same electrode in 50 nM Pb^{2+} revealed a decrease in the Pb^{2+} signal of 3% per regeneration. This could suggest that the SAM had begun to deteriorate with use, possibly due to potential cycling down to −0.44 V. Evidence that the decline in current is due to Pb^{2+} being lost due to deterioration of the SAM at the high cathodic potentials employed during the measurement process, rather than due to slow desorption of the complexed Pb^{2+} from the electrode surface was obtained as follows. After accumulation of the Pb^{2+}, the MPA–angiotensin/Pb^{2+} modified electrode was incubated in the measurement solution for the period of time that it normally takes to run a CV whereupon an actual CV was measured. When this waiting procedure was performed there was no difference in current to when there was no waiting procedure, thus confirming the decrease in current is not a function of the time the electrode is in the measurement solution.

To further confirm that the diminishing lead signal is due to deterioration of the SAM, the electrochemical stability window of MPA was investigated. Shown in Fig. 2 is the cyclic voltammogram of an MPA modified gold electrode scanned at a starting potential of 0 to −0.8 V and back for three cycles at a scan rate of 0.1 V s^{-1} in 50 mM ammonium acetate buffer (pH 7.0) and 50 mM NaCl. In the first cycle, the MPA desorption peak occurs at −0.58 V with a smaller reoxidation peak at −0.44 V from partial re-adsorption of MPA. In the second scan, MPA desorbs at a more positive potential of −0.5 V, the difference in peak potential due to the greater ease in desorption of a less densely packed SAM [48].

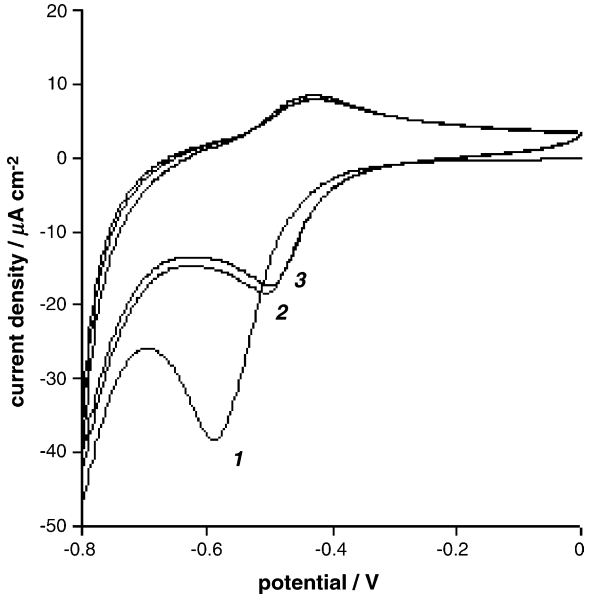


Fig. 2. Cyclic voltammogram in 50 mM ammonium acetate buffer (pH 7) containing 50 mM NaCl showing the region of desorption of MPA modified electrodes. Scan rate: 0.1 V s^{-1}.

Importantly, although the desorption peak of MPA occurs in the −0.5 to −0.6 V range, MPA actually begins to desorb before −0.4 V. For the voltammetry experiments carried out earlier, the reduction wave of Pb^{2+} occurred at −0.36 V, which made it necessary to scan to at least −0.44 V.

Even though the regeneration of the modified electrode was poor, the reproducibility for a single use of MPA–angiotensin I modified electrodes was remarkably good. So this sensor may be better as a single-use disposable device. The dependence of the OSWV current density at an MPA–angiotensin I modified electrode on the concentration of Pb^{2+} in the accumulation solution was calibrated using four modified electrodes prepared on different days (Fig. 3). Pb^{2+} was accumulated at the modified electrodes in ammonium acetate for 10 min before OSWV measurements. The relation between current and concentration is clearly non-linear but does follow a 'Langmuir-like' relation despite the system being under kinetic control rather than at equilibrium. The Langmuir equation, which does however provide an indication of the sensitivity and dynamic range of the sensor, is:

$$I = \frac{I_\infty KC}{1 + KC} \quad (1)$$

where I_∞ is the limiting current density corresponding to saturation of the surface by lead ions, and K the affinity equilibrium constant for the metal binding to the peptide. The data for the different Pb^{2+} concentrations and peak currents were fitted to Equation (1) using Solver in Excel (Microsoft, Office 2000, Seattle, USA), rather than a linear form, to preserve the expected normal distribution of the quantity I. This allowed calculation of standard deviations of the estimates of the parameters to be made: $I_\infty = 15 \pm 1\ \mu A\ cm^{-2}$ (95% confidence interval on fitted parameter) and $K = 0.018 \pm 0.003\ nM^{-1}$. The value of I_∞ gives an indication of the sensitivity of the sensor which has implications for the detection limit whilst the value of K is indicative of the affinity of the peptide for the metal ion in question and hence the concentration range of the biosensor. As far as we are aware there is no published affinity constants for angiotensin I with either Zn^{2+} or Pb^{2+}. However, the value of K is more than four orders of magnitude lower than the value we determined for the tripeptide Gly–Gly–His, bound to an electrode surface in the same way, for copper [18], As a consequence of the lower affinity constant for MPA angiotensin/Pb^{2+} the final sensor is expected to operate in a higher concentration range with a higher detection limit than the MPA–Gly–Gly–His/Cu^{2+} modified electrode. The lowest concentration of Pb^{2+} detected for the angiotensin I modified elecrode was 1.0 nM as determined by OSWV, which is well below the Australian drinking water guideline level of 48 nM (10 ppb)[2].

The use of a non-linear calibration equation gives a wider dynamic range for the electrode (see Fig. 3), compared to a pseudo-linear range that can be drawn between about 2 and 3 nM.

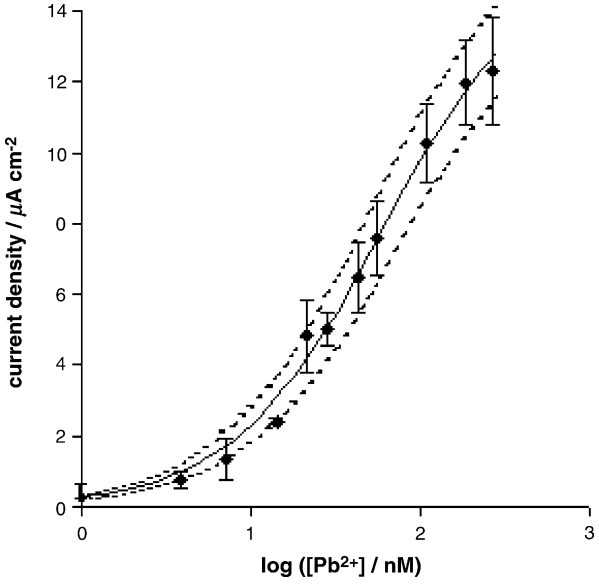


Fig. 3. OSWV peak current density for the reduction of Pb^{2+} at an MPA–angiotensin I modified electrode as a function of Pb^{2+} concentration. Error bars are ±1 standard deviation of the current densities of four individual electrodes. Experimental conditions as given for Fig. 1(b). Solid line is a fit to Equation (1) with $I_\infty = 15 \pm 1\ \mu A\ cm^{-2}$ (95% confidence interval on fitted parameter) and $K = 0.018 \pm 0.003\ nM^{-1}$. Dashed lines are 95% confidence intervals on the current density arising from the uncertainties in the regression coefficients.

3.2. Detection of Pb^{2+} using TA–angiotensin I modified gold electrodes

For developing a sensor, which satisfies the requirement of long-term use and stability, TA–angiotensin I modified gold electrodes were investigated. TA modified SAMs consist of six carbon atoms from the thiol to the carboxyl group and, in conjunction with its disulfide giving two attachment points to the gold electrode should offer higher stability [49,50]. Illustrated in Fig. 4 is the cyclic voltammogram of a TA modified electrode in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl cycled between 0 to −0.9 V at a scan rate of $0.1\ V\ s^{-1}$. TA begins to desorb at −0.45 V, peaking at −0.74 V. A smaller reoxidation peak is evident at −0.66 V. Upon further scanning, the TA desorption peak shifts slightly positive (about 0.03 V). Further scans show minor changes in peak size and position. These results compare well to those reported by Cheng and Brajter-Toth in neutral solutions [51]. Comparing the results of MPA and TA SAMs, TA offers an extra 0.10–0.15 V for stability towards reduction which proves essential for stable Pb^{2+} detection.

Shown in Fig. 5(a) is the cyclic voltammogram of the TA–angiotensin I modified electrode upon accumulation in 50 nM Pb^{2+} for 10 min at a scan rate of $0.1\ V\ s^{-1}$. The voltammogram shows well-defined lead reduction peaks and is stable over five successive cycles. The redox transition occurs at −0.17 V, which is 0.125 V more positive than the transition for the MPA–angiotensin/Pb^{2+} species. Further evidence that the redox transition is two electrons comes

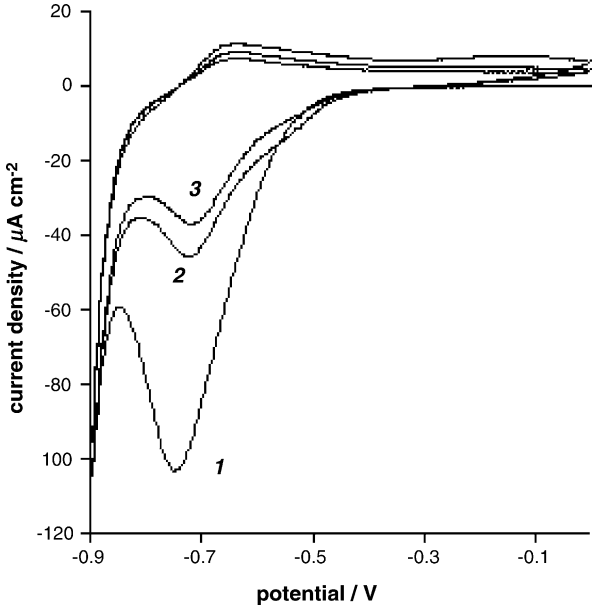


Fig. 4. Cyclic voltammogram in 50 mM ammonium acetate buffer (pH 7.0) containing 50 mM NaCl showing the region of desorption of TA modified electrodes. Scan rate: 0.1 V.

from plots of $E^{0\prime}$ versus log([Pb^{2+}]) which gave a slope of $+0.026 \pm 0.004$ V. The theoretical slope is derived from the Nernst equation, which is +0.0285 V (+0.059/n V where n is the number of electrons transferred). Measurements of cathodic OSW voltammograms at the modified electrode under the same accumulation conditions (Fig. 5(b)) resulted in a mean current density of 3.2 μA cm^{-2} ($s = 0.3$ μA cm^{-2}, $n = 4$ electrodes). Although this is a smaller current than MPA–angiotensin modified electrodes, for the same Pb^{2+} concentration the peak is sharper and the baseline has less of a slope at negative potentials. The rate of electron transfer of lead was calculated using the Laviron equation in the case where $\Delta E_p > 0.2/n$ V [52]. For TA–angiotensin modified electrodes the electron transfer rate constant was $k_{ET} = 1.3\ s^{-1}$ ($s = 0.2\ s^{-1}$, $n = 3$ electrodes) which is not significantly different from MPA–angiotensin modified electrodes ($k_{ET} = 1.7\ s^{-1}$ ($s = 0.3\ s^{-1}$, $n = 3$ electrodes), $p = 0.11$).

Regeneration and reuse of the TA–angiotensin I modified electrode by removing bound Pb^{2+} and then reaccumulation gave promising results. There was no apparent loss in signal (<1%) after five regenerations. This result highlights the importance of using a SAM which has two sulfur anchoring points over MPA as its higher stability offers longer term use without significant reduction in the ability to probe redox species electrochemically (as evidenced by the similarity in k_{ET} for MPA–angiotensin I and TA–angiotensin I modified electrodes).

The OSWV current density at a TA–angiotensin I modified electrode was calibrated as a function of the concentration of Pb^{2+} in the accumulation solution. Pb^{2+} was accumulated at the modified electrodes in ammonium acetate for 10 min before OSWV measurements. The data were fitted in

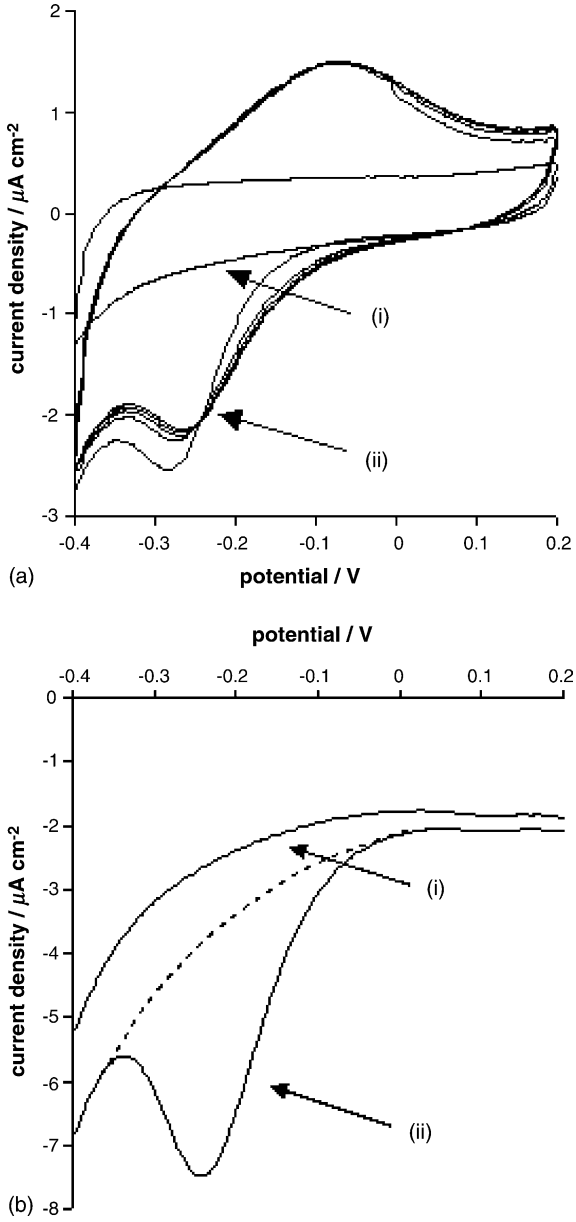


Fig. 5. (a) Cyclic voltammograms of a TA–angiotensin I modified gold electrode in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl (i) before accumulation of metal ions and (ii) after accumulation in 50 nM Pb^{2+} in 50 mM ammonium acetate (pH 7.0) for 10 min. Scan rate: 0.1 V s^{-1}. (b) Osteryoung square wave voltammograms of a TA–angiotensin I modified gold electrode in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl (i) before accumulation of metal ions and (ii) after accumulation in 50 nM Pb^{2+} in 50 mM ammonium acetate (pH 7.0) for 10 min.

a similar manner to the Pb^{2+} complexed MPA–angiotensin modified electrodes, resulting in $I_\infty = 8.2 \pm 0.7$ μA cm^{-2} (95% confidence interval on fitted parameter) and $K = 0.013 \pm 0.003\ nM^{-1}$. The value of K is very similar to MPA–angiotensin as expected since the same ligand is used to complex to Pb^{2+} ions. The relationship between concentration and current density, which would be used to estimate a concentration from a measured OSWV peak is shown in Fig. 6 with 95% confidence intervals on the estimate. The

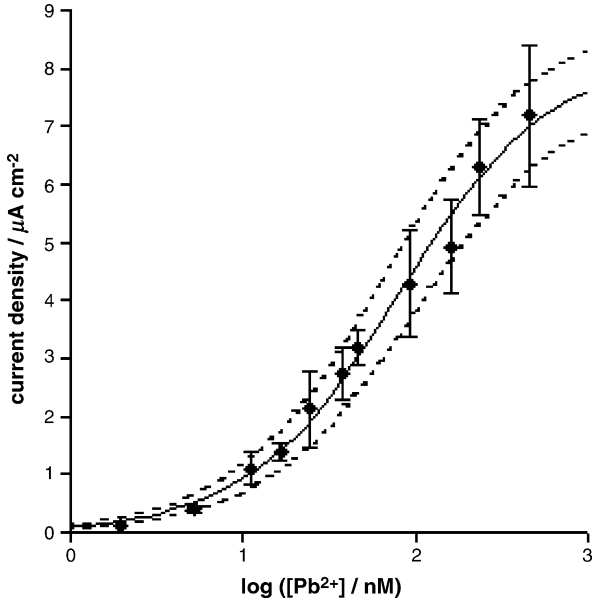


Fig. 6. OSWV peak current density for the reduction of Pb^{2+} at a TA–angiotensin I modified electrode as a function of Pb^{2+} concentration. Error bars are ±1 standard deviation of the current densities of four individual electrodes. Experimental conditions as given for Fig. 1(b). Solid line is a fit to Equation (1) with $I_\infty = 8.2 \pm 0.7\,\mu A\,cm^{-2}$ (95% confidence interval on fitted parameter) and $K = 0.013 \pm 0.003\,nM^{-1}$. Dashed lines are 95% confidence intervals on the current density arising from the uncertainties in the regression coefficients.

lowest measurable lead concentration was 1.9 nM which is higher than that found with MPA–angiotensin modified gold electrodes but a wider dynamic range, up to 460 nM, was achieved. The most noticeable difference between the two calibration relations is the higher saturation current of the MPA–angiotensin modified electrodes ($15 \pm 1\,\mu A^{-2}$) compared to TA–angiotensin ($8.2 \pm 0.7\,\mu A^{-2}$). The higher current is attributed to the larger surface coverage of an MPA SAM compared to a TA SAM which results in a larger proportion of angiotensin I attached to the surface. Reductive desorption of MPA and TA modified electrodes in 0.5 M KOH and calculation of the charge passed in the voltammograms results in a surface coverage of $0.78\,nmol\,cm^{-2}$ ($s = 0.06\,nmol\,cm^{-2}$, $n = 4$ electrodes) and $0.54\,nmol\,cm^{-2}$ ($s = 0.08\,nmol\,cm^{-2}$, $n = 4$ electrodes), respectively.

Overall, TA–angiotensin I modified electrodes are much better for Pb^{2+} detection in terms of stability and reuse. Its inferior sensitivity and detection limit represents a minor compromise relative to MPA–angiotensin I as the TA–angiotensin I is more than adequate for lead drinking water standards. Compared to previous Pb^{2+} sensors, the detection limit offered by TA–angiotensin I and MPA–angiotensin I modified electrodes are an order of magnitude better than the fluorosensor developed by Li and Lu [8] with a detection limit of 10 nM Pb^{2+} and the cysteine desulfhydrolase tissue biosensor developed by Kremleva et al. [9] of 30 nM Pb^{2+}. The enzyme biosensor developed by Veselova and Shekhovtsova [10] had a lower Pb^{2+} detection limit of 0.10 nM but this biosensor encountered selectivity problems with other metal ions.

3.3. Interference studies

The interference effect of other metal ions with the Pb^{2+} signal was addressed for the TA–angiotensin I modified electrodes. The interferents chosen for this study were metal ions that are commonly found in natural samples and that could possibly compete for angiotensin binding sites. The metal ion need not be electroactive as we are only interested in how the Pb^{2+} peak current density changed in the presence of interferents. The selected metal ions were Cu^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Cr^{3+} and Ba^{2+}. For the determination of the effects, a two-level Plackett–Burman experimental design [53] was constructed consisting of eight experimental runs with all samples containing 106 nM Pb^{2+} plus a low level (−1, nominal 50 nM) or high level (+1, nominal 500 nM) of each interferent. Table 1 shows the contrast coefficients for the design. Since the design can only be used with $(4n - 1)$ variables, a dummy factor (one where the change is known to have no effect) was introduced as a measurement of the standard deviation of the factor effects. The effects of a factor (the concentration of metal ion interferents) were calculated by summing the responses multiplied by their contrast coefficients, +1 or −1, and then dividing by half the number of runs. The calculated effect is the effect upon changing the metal ion concentration from low to high. Its significance was determined by constructing a Rankit plot, which for normally distributed data should fall on a straight line close to zero if the measured effects are due to random errors. Outliers from this line indicate significant effects. In the absence of any interferents, the cathodic OSWV current density was $4.5\,\mu A\,cm^{-2}$. Fig. 7 is the Rankit plot of the effects of the seven variables (six potential interfering metal ions plus a dummy variable). All the effects, including that of the dummy factor, fell on a straight line. The fact that no interference was observed from Zn^{2+} provides a strong indication that Pb^{2+} binds more strongly to the angiotensin peptide, with a higher affinity constant than Zn^{2+}. As part of a separate experiment, the effects of Hg^{2+} and Ag^{+} were investigated in a two-level experimental design. This was carried out separately since these metal ions are expected to interfere with the Pb^{2+} signal. Hg^{2+} in

Table 1
Two-level experimental design for interference studies of lead complexation to TA–angiotensin I modified electrodes

Run	Cu^{2+}	Zn^{2+}	Ni^{2+}	Cd^{2+}	Cr^{3+}	Ba^{2+}	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

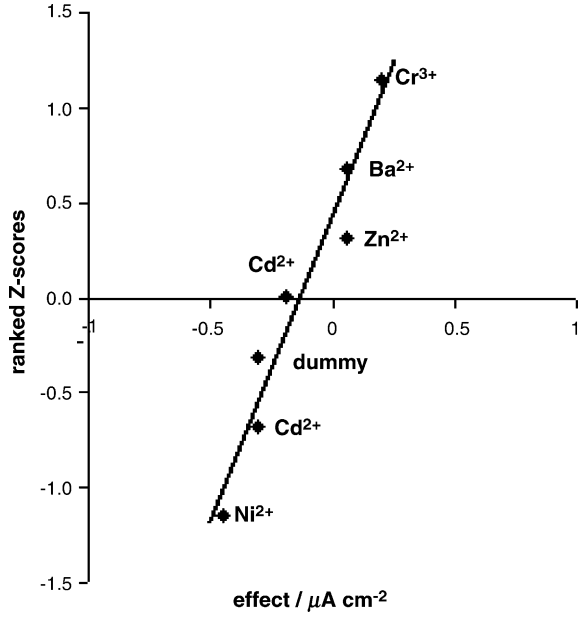


Fig. 7. Rankit plot for the effects of interferents from a Plackett–Burman 7-factor experimental design. Experimental conditions as given for Table 1.

environmental samples can be a significant problem since it can oxidize thiols and disrupt the SAM electrode structure [54]. When the concentration of interferents was changed from low (50 nM) to high (500 nM), Hg^{2+} and Ag^{+} affected the Pb^{2+} signal by $-0.8\,\mu A\,cm^{-2}$ (18%) and $-0.3\,\mu A\,cm^{-2}$ (7%), respectively. The large negative effect for Hg^{2+} may be considered to be significant although the levels chosen for this design are significantly higher than those generally found in natural waters [55].

3.4. Analytical application

The analytical application of TA–angiotensin I modified electrodes was demonstrated in the determination of Pb^{2+} in a lake water sample collected from Lake Bedford (Sydney Park, Australia). The sample was diluted five-fold so as to allow the concentration of Pb^{2+} to fall within the dynamic range of the TA–angiotensin I calibration curve. The concentration of Pb^{2+} in lake water, after dilutions were accounted for, is 6.8 ± 1.2 nM (95% confidence limits). The analytical result obtained is much lower than that measured by ICP-MS of 72 nM presumably due to the differences in speciation of both techniques. The TA–angiotensin modified electrode gives an indication of free Pb^{2+} ions or weakly bound lead complexes and hence provides a more reliable measurement of toxicity than ICP-MS, which measures the total metal content.

4. Conclusions

MPA–angiotensin I and TA–angiotensin I modified gold electrodes have been used to detect low levels of Pb^{2+} ions, the latter electrode giving a more stable lead signal as demonstrated by cyclic voltammetry of complexed Pb^{2+} in metal-free buffer solution. The superiority of TA–angiotensin modified electrodes was also evidenced by its longer storage life and reusability, which was achieved by regenerating the electrodes to Pb^{2+}-free using $HClO_4$. In the study of interferences, Hg^{2+} was the only ion found to significantly affect the Pb^{2+} current. Overall, the work demonstrated how the ease in changing a modification step from an MPA SAM to a TA SAM provided a more stable and reliable platform for the immobilization of peptides.

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