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Peptide Modified Electrodes as Electrochemical Metal Ion Sensors

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Abstract
Sensors for the detection of metal ions are of considerable importance for enabling the monitoring of environmental samples for metal ion contamination directly in the field. This review outlines the use of peptides and amino acids as the recognition element of electrochemical sensors for metal ion detection. Initially the complexation of metals by peptides is discussed followed by the immobilization of peptides on electrode surfaces. Subsequently, the application of peptide modified electrodes for detecting metals is reviewed and finally challenges and future prospects are outlined.

Keywords: Peptide modified electrodes, Metal ion sensors, Self-assembled monolayers, Environmental monitoring

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1. Introduction

Certain metals are essential for life processes although many are also toxic due to their interference with the function of metalloenzymes and key biochemical pathways [1]. The accumulation of heavy metals, such as lead, cadmium and mercury, in living organisms can result in an impairment of function, or in extreme cases, death. For example, the European Union guideline level for lead in drinking water is set at 10 ppb [2] since severe effects can be observed upon exposure to higher levels. The toxicity of lead is derived from its ability to interfere directly with calcium signaling, by substitution since lead has a similar ionic radius to Ca^{2+} [3], and the inhibition of several zinc enzymes [3–7]. However, numerous toxicological studies have shown that even essential metals can exhibit toxic effects at elevated levels [3]. For example, copper is an essential metal with an estimated daily requirement of 2–3 mg, yet high concentrations may result in gastrointestinal symptoms such as nausea, abdominal pain and vomiting. Therefore, the ability to monitor concentrations of metal ions becomes important in providing a means to assess toxicity levels.

At present, the routine determination of metal ions is carried out using instrumentation such as atomic absorption spectroscopy (AAS) and inductively coupled plasma-mass spectrometry (ICP-MS). These provide highly reliable measurements on total metal concentrations and, in the case of ICP-MS, can measure several metals at one time. The drawbacks of these instrumental methods are the high cost of purchasing and running the instruments and the fact that they are laboratory based. In-the-field monitoring of metals is required so that the appropriate action can be taken immediately if guideline levels are exceeded. Most metals are commonly found in natural waters and can be introduced through the dissolution of minerals and ores, or from industrial effluent and atmospheric deposition [8]. Thus, field instruments are required so that measurements can be carried out in real time without the need for samples to be taken back to the laboratory for analysis.

Biosensors are a possible solution to the portability issue [9–11]. Typically a biosensor is fabricated by immobilizing a biological recognition molecule, such as an antibody, enzyme, sequence of DNA or whole cells over a signal transducer, such as an electrode or optical device, to give a reagentless, portable, solid state sensor. Apart from biosensors being portable and capable of real time in-field monitoring, the use of biological molecules to detect metals could perhaps enable biosensors to provide an indication of how metal ions interact with a particular organism. Such a possibility presents new opportunities for metal ion detection methods which could give an indication of the bioavailable metal ion concentrations as distinct from total metal ion levels; a possibility which is beyond the scope of ICP-MS, AAS and other methods which do not employ biological molecules.

The development of biosensors for metal ions has become an active research area of late with bacteria [12–14], DNA [15], enzymes [16–18], other proteins [19–21] and peptides [4, 22–39] all having been exploited as the biorecognition molecule in electrochemical and optical biosensors. These approaches have illustrated an enormous promise but also often tend to suffer from insufficient selectivity. Enzyme biosensors for metal ions which employ alkaline phosphatase as the biorecognition molecule highlight the advantages and disadvantages of many protein based biosensors for detecting metal ions developed thus far. Assays based on the inhibition of alkaline phosphatase with Pb(II) [40] and the activation of the apo-enzyme by Zn(II) [41] show sub-ppb

detection limits, thus demonstrating the exquisite sensitivity that can be achieved. However, alkaline phosphatase is also inhibited by Zn, Bi, Be, Cu, and As [18, 40–42]; thus illustrating the selectivity problem.

The problem of a number of metal ions inhibiting alkaline phosphatase indicates that the peptide sequences that can make up the enzyme can bind to a large range of metal species. On the other hand, the activation assays employing zinc and apo-alkaline phosphatase are very selective for that metal ion which illustrates that certain peptide motifs can be highly selective for particular metal ions. Such selectivity is also illustrated by the multitude of enzymes that have specific metal ions as cofactors. As a consequence, the incorporation of peptides into biosensors is expected to have considerable potential in the development of metal ion based biosensors. Peptides have been used as solution based metal ion sensors using fluorescence to transduce the binding of the metal ion [4, 5, 43]. For example, Godwin and co-workers have used zinc fingers as highly selective fluorosensors for Zn^{2+} [4] by modifying the zinc finger consensus peptide with fluorescein as the donor and lissamine as the acceptor. In the absence of Zn^{2+}, the peptide is unfolded and the dyes are far apart whereas in the presence of Zn^{2+}, the peptide changes its conformation so that the fluorophores are closer together increasing the amount of energy transfer. Furthermore, the collection of natural, and synthetic, amino acids act as an alphabet from which a myriad of ligands with varying selectivities can be synthesized.

The transition from using peptides in solution to detect metal ions to using peptides as the basis of metal ion biosensors is the immobilizing of the peptides onto a signal transducer to give a solid state device. This review will highlight biosensors which utilise peptides at electrode surfaces as a basis of biosensors. Firstly, the basics of metal ion binding will be discussed followed by the complexing of metals by peptides. Subsequently, approaches to modifying electrode surfaces with peptides will be discussed, followed by a discussion of our own research motivated towards using peptide modified electrodes for the detection of a suite of metal ions. Finally we will outline the future prospects and challenges facing the application of peptide modified electrodes for detecting metals and include some of our approaches to solving these challenges.

2. Metal Complexation to Peptides

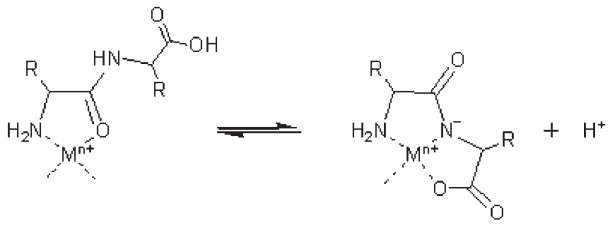
Some aspects of metal complexation important in the context of using peptides for the detection of metals are outlined in this section, although for a more comprehensive discussion the reader is directed to reviews by Sigel and Martin [44] and Gurd and Wilcox [45].

Peptides possess a variety of donor atoms through the peptide backbone and amino acid side chains for complexation to metal ions. The complexes formed can exist in a variety of conformations that are dependent on the pH of the environment and the concentration of both the peptide and metal ion.

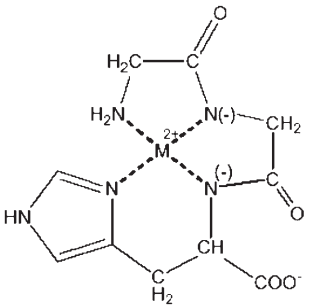
In simple peptides with non-coordinating side chains, binding occurs through the peptide backbone. Two alternative binding modes involving the amino group and amide oxygen or the amino group and the amide nitrogen respectively are illustrated in Scheme 1 for a dipeptide with non-coordinating side chains [44]. Chelation involving the amino group and the amide nitrogen also allows the carboxyl oxygen to be involved, thus producing a more stable complex as illustrated in the structure to the right of the equilibrium sign in Scheme 1. In view of this binding mode, coordination of the metal via the amide nitrogen needs to be capable of substituting for the nitrogen bound hydrogen. Therefore, the concentration of hydrogen ions not only controls the extent of hydrolysis of the hydrated metal ion but also competes with the metal ion for the ligand. Hence metal complexation using peptides is dependent on the pH environment.

If the dipeptide is extended to a tri- or tetrapeptide or even longer, significantly stronger binding would be observed as each amide nitrogen along the peptide backbone becomes involved in the coordination. This can be illustrated with Gly-Gly-His in which the coordination of Cu^{2+} involves the successive deprotonation of the amide nitrogens and the imidazole nitrogen at increasingly higher pH values until a 4 N tetradentate species is formed at pH 7 (Scheme 2) [46–49]. In contrast, the uptake of cadmium ions by poly-L-cysteine is independent of the pH environment (over the pH range of 1.0–7.0) due to the high pK_a value of the coordinating thiol side group of cysteine (see Table 1) [25].

The binding of metals to the backbone of peptides is reasonably nonselective and explains the relatively poor selectivity of some protein based sensors for metal ion detection. However, the examples above with Gly-Gly-His and poly-L-cysteine indicate that the different donor atoms on the side chains of the amino acids are pivotal in giving peptides selectivity for particular metals. Polar side chains which might act as ligands for metal ions are presented in Table 1 [50]. The imidazolium group of histidine is the most important binding site for zinc, copper and other ions in the case of serum albumin [51] whereas cysteine is perhaps the



Scheme 1. Metal ion complexation to a dipeptide with non-coordinating side chains. The structure on the left involves coordination of the metal through the amino group and amide oxygen. A more stable complex exists if the metal coordinates through the amino group, amide nitrogen and carboxyl oxygen as illustrated in the structure on the right.

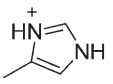
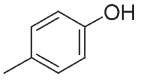
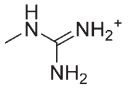


Scheme 2. Cu^{2+} complexation by Gly-Gly-His.

most effective ligand for binding heavy metals with often very high metal-sulfur affinities. The order of affinity for sulfur is: $Hg^{2+} \approx Ag^{+} \gg Cu^{2+} > Pb^{2+} \approx Cd^{2+} > Zn^{2+} \gg Ca^{2+} \approx Mg^{2+}$ [51].

Apart from the guanidinium, aliphatic hydroxyl and amide groups, each of the polar side groups in Table 1 are involved in complex formation with at least some metals [50]. The exclusion of the guanidinium, aliphatic hydroxyl and amide groups in complex formation is due to the competition with hydrogen ions. These three groups all possess high pK_a values and therefore protons cannot be displaced from these groups at neutral or low pH. Instead, the charged guanidinium group plays a role in complex stability. Studies by Tanford and Shore [52] have shown that the side chain of arginine combines with Co^{2+} (log K_1 value of 3.87) to a lower extent than alanine with Co^{2+} (log K_1 value of 4.27) with the difference in log K_1 values attributed to the electrostatic repulsion between the guanidinium group and Co^{2+} [52]. In contrast, amino acids containing aromatic side chains such as Tyr and Phe can contribute to the stability of a complex through hydrophobic interactions or ring stacking.

Table 1. Polar side chains in peptides and their corresponding pK_a values [50].

Side chain	Side chain structure	Approximate pK_a
Carboxyl		4
Imidazolium		6
α-Ammonium		6 – 8
ε-Ammonium		10
Phenolic		10
Thiol		10
Guanidinium		12
Hydroxyl		≥14
Amide		≥14

To predict metal-ligand complexation, the A-B classification scheme has commonly been used [53]. Class A (hard) metals include K^{+}, Li^{+}, Na^{+}, Ba^{2+}, Ca^{2+}, Mg^{2+} and Al^{3+}. They are non-polarisable, highly stable and form weak complexes via electrostatic bonding. The ligand-binding preferences of these metals decrease in the following order: O > P > N > S. In other words, these metals bind preferentially to carboxylate found on peptides and amino acids and phosphate groups on DNA. For class B (soft) metal ions, there is a reversal in ligand-binding preference to class A metals. These metals include Ag^{+}, Hg^{2+}, Cd^{2+} and Au^{+} and are all non-essential and mostly toxic. For example, amino acids bearing N donor ligands in their side chains (in particular, histidine, lysine and arginine) and the S donor ligand (cysteine) have strong binding preferences for class B metals. Metals having ligand-binding characteristics that are intermediate to those of group A and B are known as borderline metals. This class includes Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+} and Zn^{2+}. Cu^{2+} and Pb^{2+} may be classified as borderline or class B metals.

The effect of amino acid side chains bearing hard or soft ligands can be illustrated with the glutathione tripeptide, γ-Glu-Cys-Gly. Glutathione presents eight possible coordination sites: two carboxyls, one thiol, one amine and two pairs of carbonyl and amide donors [54]. It is highly versatile as a ligand since it can form complexes with both hard and soft metal ions. The soft thiol group of cysteine has a high affinity for soft metal ions, Cd^{2+}, Ag^{+} and Hg^{2+}, whereas hard metals interact primarily with the glutamic acid moiety.

The steric effect also plays an important role and this can be illustrated with glycine and L-leucine. There is little difference between the acidity of the α-ammonium group of these amino acids, yet the formation constant of the L-leucine complex with Mn^{2+} is lower than that of glycine with Mn^{2+} [55]. This difference must be attributed to the size of the aliphatic side chain. Although this study was carried out using single amino acids, the effect with peptides should be similar.

With at least twenty amino acids available, arranged in any particular order or length, peptides present an almost infinite number of ligands for binding metal. The combination of steric effects, presence of coordinating side chains in a peptide sequence, hard-soft character of a metal ion and the pH environment makes it difficult to precisely predict the relative affinity of a given ligand for a particular metal ion. Hence experimental approaches are still the best way of determining the relative affinity and selectivity of a peptide for a particular metal. Methods which efficiently allow the screening of peptides for metal binding capability are outlined in the next section.

3. Identification of Selective Peptide Ligands

To identify appropriate peptide ligands for the sensing of specific metal ions, attention can be turned to techniques such as mass spectrometry (MS) [56, 57], nuclear magnetic resonance (NMR) [58, 59], X-ray photoelectron spectroscopy (XPS) [33, 60, 61] or pH titrimetry [47] which have been commonly used for the study of peptide-metal complexation. These techniques provide information concerning the stoichiometry of the complexes formed and groups that are involved in coordination. Although a lot of structural information can be gathered from these techniques, they require selecting a known (and typically small) variety of peptides for analysis and so are better suited for the characterization rather than identification of selective peptide ligands.

A relatively new and powerful technique for the identification of peptides that selectively bind metal surfaces are phage display peptide libraries [62]. Phage display libraries are a combinatorial biology protocol where a large random library of peptides of the same number of amino acids but different sequence are generated by inserting randomized oligonucleotides within certain genes encoded on phage genomes which leads to incorporation of random polypeptide sequences within the protein coat of the phage. To identify polypeptides selective for a certain metal surface the metal surface is exposed to a phage display library for several hours, followed by washing away the unbound phages and recovering the metal surface attached phages by elution with an acidic buffer [62]. The exposure and elution process is repeated several times. The collection of phages is then amplified by infecting the host bacterium *Escherichia coli* (*E. coli*). The output phage is then used for further rounds of selection in a process also known as biopanning. One early demonstration of this technique was by Brown who isolated metal binding polypeptides using a bacterial combinatorial library with gold powder as the target [63]. The gold binding polypeptides contained several direct repeats of identical peptide units and were selected from a population of approximately five million polypeptides. Since then, selective peptides have been isolated for platinum [64], palladium [64], titanium [65], silver [66, 67] and cadmium [68]. The majority of these studies investigate solid metals and hence the relationship with metal ion detection may not be clear. However, Bülow and co-workers identified the cadmium binding peptide, His-Ser-Gln-Lys-Val-Phe, using a metal chelating Sepharose gel with an input of 10^{12} phages [68]. Expression of the hexapeptide on the surface exposed area of the outer membrane protein OmpA of *E. coli* showed an increased tolerance to cadmium toxicity due to reduced cadmium uptake. This peptide has subsequently been used as the selective recognition component of cadmium metal ion sensors [29, 69].

The identification of these selective peptide ligands can then be exploited as biomimetic ligands in the sensing of metal ions using solid state devices.

4. Peptides on Electrode Surfaces

The solution based studies of peptide-metal complexes suggest there is considerable potential for using peptides to detect metals. However, peptides need to be immobilized on an electrode surface for ease in performing in-field measurements. An important consideration in the modification of peptides at electrode surfaces is suitable attachment chemistry with control over the conformation and spacing of the ligands to allow the metal ion to access the binding site. This requires consideration of the choice of electrode material in terms of the chemical modifications which can be applied to the substrate as well as the working potential range, surface roughness, cost and environmental friendliness. There are a variety of different approaches to peptide immobilization with physisorption and covalent attachment to a self-assembled monolayer surface the most prevalent in the literature. These two alternative immobilization procedures essentially follow the two phases of using peptides in the detection of metal ions in electroanalytical chemistry.

Methods which rely on the physisorption of molecules onto electrode surfaces were popular in the 1980's, particularly using mercury electrodes. For example, Fogg and co-workers utilized the strong adsorption of polymers onto mercury electrodes for sensing Cu^{2+} [22]. The poly-L-histidine film was modified at a hanging mercury drop electrode and Cu^{2+} at concentrations between 5 nM and 0.4 μM was accumulated for 2 min at −0.4 V versus Ag|AgCl followed by differential pulse adsorptive stripping voltammetry. The high affinity of Cu^{2+} for the polymer film was through the binding to three imidazolium groups and an amide nitrogen and this was not affected by the presence of micromolar levels of ethylenediamine tetraacetic acid, Cr^{3+}, Pb^{2+}, Ni^{2+}, Cd^{2+} or Mn^{2+}. However, the use of hanging mercury drop electrodes, which is highly toxic, is not compatible with a solid state analytical device.

The first example of peptides self-assembled onto electrode surfaces was by Takehara et al. who used glutathione (γ-Glu-Cys-Gly) self-assembled monolayers (SAM) on gold electrodes as ion gates for detecting lanthanide ions [26]. The self-assembly of thiols on gold is a useful strategy since monolayer formation is essentially instantaneous and the SAM can be tuned with different surface functionalities which can be further modified with more complex molecules. The attractive feature of glutathione is that it presents itself with a thiol group through the cysteine amino acid which is available for the direct formation of the Au-S linkage. The ion-gate behavior of the glutathione modified gold electrode was studied by cyclic voltammetry using ferricyanide as the redox probe. The monolayer functions as an "on/off" switching gate for the permeation of ions where in the absence of lanthanide ions and at a pH greater than 5.7, a barrier of negatively charged carboxylate groups prevents the movement of redox active species to the electrode (Fig. 1a). Upon metal binding to the carboxylate groups, the authors hypothesize the peptide changes its conformation to a more contracted form, thus opening up channels for ions to access the electrode surface (Fig. 1b).

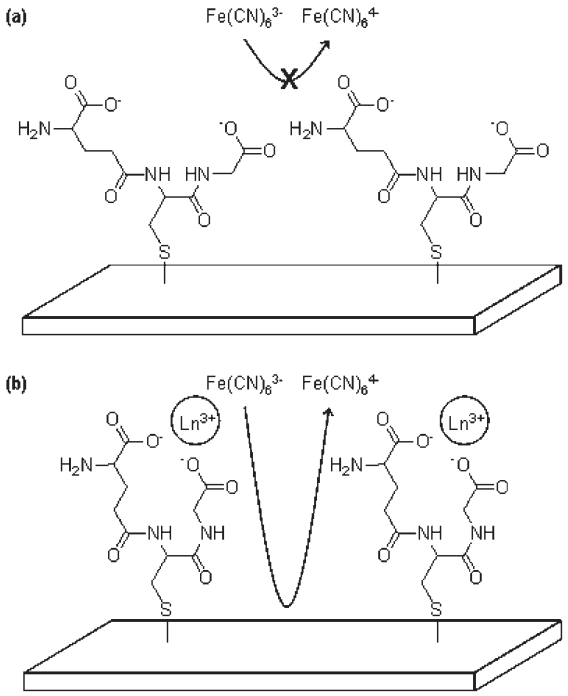


Fig. 1. Schematic representation of GSH self-assembled directly on a gold electrode a) in the absence of lanthanide ions and b) in the presence of lanthanide ions.

The response to lanthanides was $La^{3+} > Eu^{3+} > Lu^{3+}$ with a La^{3+} detection limit of about 1 μM. The glutathione monolayer as an ion gate is highly sensitive to the concentration of lanthanide ions and the sensitivity is about three orders of magnitude greater than to alkaline earth metals. Note this sensor does not rely on the redox chemistry of lanthanide ions as the transduction event which would be outside the working potential range of the glutathione modified gold electrode.

Glutathione has also been used as a recognition element for copper(II) in the μM to mM range without preconcentration by self-assembling the glutathione onto gold electrodes via the thiol side chain of the cysteine residue [27]. The transduction of Cu(II) complexation was via the reduction of the Cu(II) directly at the underlying electrode. Interestingly, the analytical performance of the peptide modified electrodes was improved by spacing the glutathione ligands apart via the formation of a mixed self-assembled monolayer of glutathione and 3-mercaptopropionic acid (MPA). The improved performance when MPA was employed indicates the importance of interfacial design on the performance of peptide modified electrodes.

The thiol side chain of cysteine has also been used to self-assemble cysteine on gold electrodes as a simple strategy for the determination of metal ions via the direct electrochemical measurement of the bound metal ion (Fig. 2). This has been carried out by the research groups of Chen [70], Arrigan [71] and Gooding [61]. The formation of a self-assembled monolayer (SAM) of cysteine leaves the carboxyl and amino groups available to form a 2:1 complex with Cu^{2+}, giving copper its preferred square planar binding

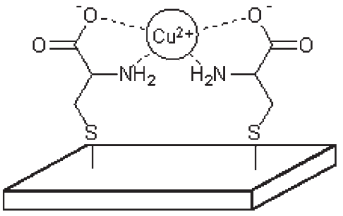
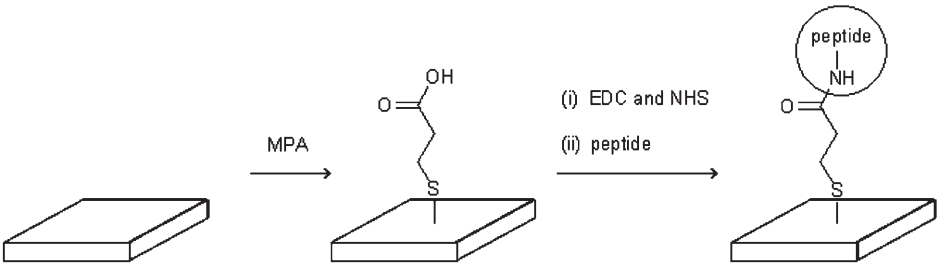


Fig. 2. Schematic representation of Cu^{2+} bound to two cysteines on a gold electrode.

geometry [61]. Detection of Cu^{2+} down to 0.39 nM was achieved by Chen and co-workers upon accumulation in copper(II) containing phosphate solution for 5 min at open circuit potential, followed by electrochemical measurements [70]. The optimal performance was at pH 5, the isoelectric point of L-cysteine. At pH values below the isoelectric point, the chelating ability is decreased due to the carboxylic acid group being protonated. At high pH values, it was proposed by Chen and co-workers that the existence of a strong interaction between the amino nitrogen and gold surface weakened the binding of the amino group to copper(II) [70]. The electrodes could be regenerated to copper free by holding the potential at 0.5 V (versus Ag|AgCl) in $HClO_4$ for 20 seconds. The advantages of direct electrochemical measurement of the analyte are that no additional redox probe is required and the redox potentials of different metal ions would occur at different potentials. The latter property could be exploited for the high discrimination and minimization of false signals from interferences. Ni^{2+} was the only ion found to affect the determination of Cu^{2+} at a cysteine modified gold electrode when present in 5000-fold excess [70].

The methodology illustrated in the last two examples is not a generic solution for modification of electrodes with peptides since the thiol side chain of cysteine is bound to the gold electrode and hence unavailable for binding the metal ion. As the thiol is an important side chain in the selective binding of class B (soft metals), such an approach to fabricating metal ion selective electrodes is somewhat limiting. To overcome this problem, a generic modification strategy was developed which allows the attachment of the amino end of any peptide sequence to a gold surface. This strategy involved the self-assembly of a carboxyl terminated SAM which could further be reacted with the amino end of a peptide [30, 31]. As a short-chained SAM will promote good electrochemical communication with a redox centre and hence high sensitivity due to the redox active centre being located close to the transducer, 3-mercaptopropionic acid (MPA) has been widely used in sensor designs [72–74]. In the fabrication of gold electrodes with peptides, MPA was first self-assembled onto a gold substrate, followed by activation of the carboxyl groups with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), and attachment of the *N*-terminus of the peptide (Scheme 3) [75]. Once attached, the binding of metal ions could be transduced by exploiting the electrochemistry of the metal. A variety of different peptides have



Scheme 3. General modification procedure for the attachment of the *N*-terminus of a peptide to an MPA SAM.

been attached to MPA modified gold electrode surfaces using this procedure including those depicted in Figure 3. The same generic method of fabricating peptide modified interfaces has also recently been exploited for metal ion detection at peptide modified surfaces using surface plasmon resonance [76].

Detection of Cd^{2+} was made possible by using glutathione as the ligand attached to an MPA SAM (Fig. 3a) [28]. The covalent attachment of glutathione to MPA was found to be superior for cadmium sensing compared to direct attachment of glutathione to the gold surface via the cysteine residue. Detection involved accumulating the peptide modified sensor in Cd^{2+} solution for 10 min at open circuit potential followed by rinsing with Cd^{2+} free solution and electrochemical measurements also in Cd^{2+} free solution. Figure 4 illustrates the voltammograms of an MPA-GSH

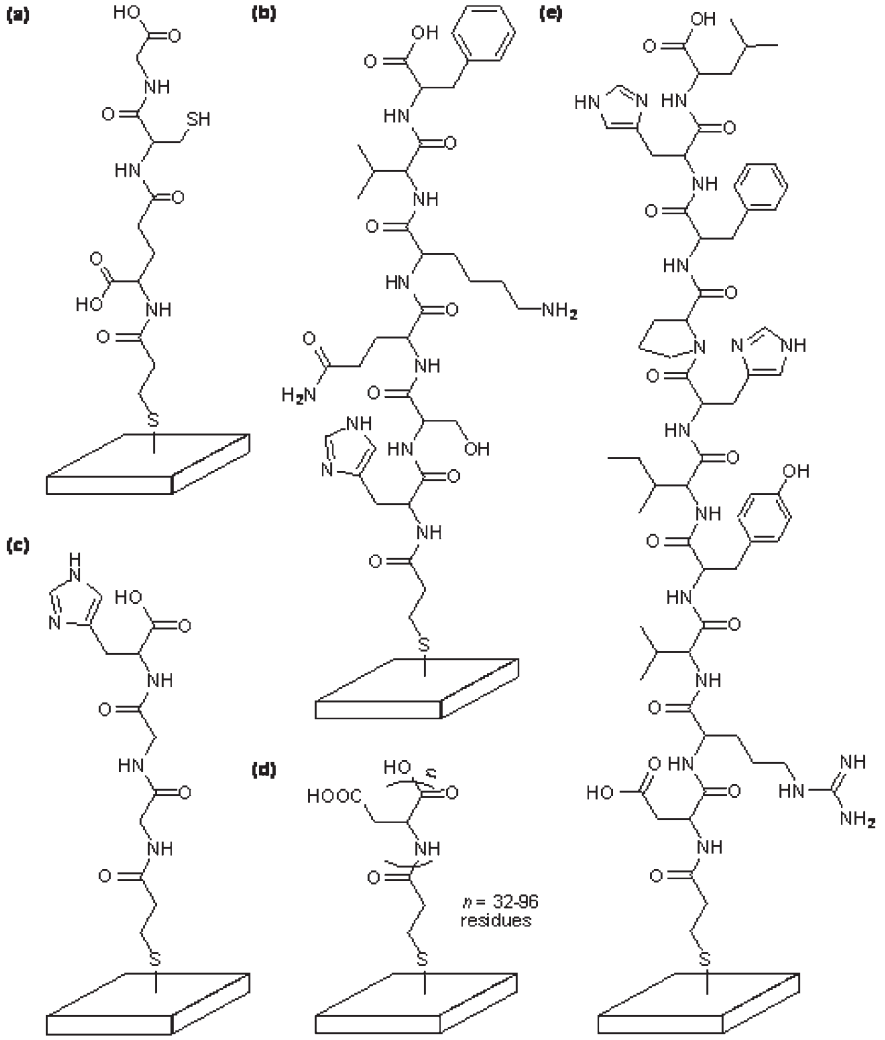


Fig. 3. Oligopeptides covalently attached to MPA SAMs at gold electrode surfaces for metal ion sensing. a) MPA-GSH for the determination of Cd^{2+}, b) MPA-His-Ser-Gln-Val-Lys-Phe for the determination of Cd^{2+}, c) MPA-Gly-Gly-His for the determination of Cu^{2+}, d) MPA-poly-L-aspartic acid for the determination of Cu^{2+} and, e) MPA-angiotensin I for the determination of Pb^{2+}.

modified electrode before and after accumulation in 98 nM Cu^{2+} for 10 min. The peptide modified electrode concentrates the metal on the electrode surface and reduction of Cd^{2+} at the electrode allows transduction. Detection of cadmium down to a concentration of 5 nM was possible using MPA-glutathione modified electrodes. However, Cu^{2+} and Pb^{2+} were found to significantly interfere with the Cd^{2+} current due to their affinity for the thiol ligand. The interference of Cu^{2+} does not come as a major surprise considering the work of Zeng et al. in using glutathione modified electrodes for detecting Cu^{2+} [27].

Improved selectivity for Cd^{2+} can be achieved by substituting the peptide His-Ser-Gln-Lys-Val-Phe (HSQKVF) for GSH. The hexapeptide, which was identified through a phage display library for Cd^{2+} [68], possesses no cysteine and hence binding is achieved without employing soft thiol ligands. The MPA-HSQKVF modified gold electrode (Fig. 3b) [29] was found to be superior to MPA-GSH [28] in terms of Cd^{2+} detection limit (0.9 nM using HSQKVF compared to 5 nM using GSH) and selectivity. Cu^{2+} did not affect the determination of Cd^{2+} using MPA-HSQKVF although Pb^{2+} was a minor interfering ion. This work is important as it demonstrates that using a carefully selected peptide, which does not contain a side-chain with affinity for a wide range of metals, such as a thiol, can give an exceedingly selective recognition element for that metal. The paper is also significant as it is the first demonstration of modifying electrodes with side-chain protected amino acids, in this case a lysine, followed by deprotection after immobilization on the electrode surface. Being able to use protected amino acids and peptides followed by deprotection on the electrode surface is important as it not only enables peptides to be immobilized with well defined orientation but also opens up the possibility of the synthesis of peptide ligands directly on the electrode surface [36]. With HSQKVF, the free amine on the lysine could also couple to the carboxylic acid moiety of the MPA modified electrode thus providing two possible orientations of the immobilized ligand peptide. To avoid coupling by the lysine residue, the amine of the lysine was protected with a *t*-butoxycarbonyl (Boc) group. After attaching the peptide to the surface the Boc group could be removed in 1% trifluoroacetic acid, 2.5% triisopropylsilane and 2% water in dichloromethane.

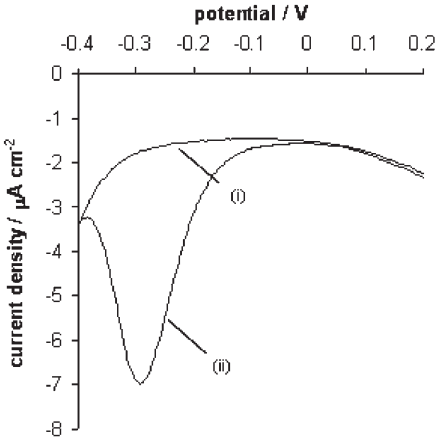


Fig. 4. Osteryoung square-wave voltammograms of an MPA-GSH modified gold electrode i) before accumulation of Cd^{2+} ions and ii) after accumulation in 98 nM Cd^{2+} for 10 min in 50 mM ammonium acetate buffer (pH 7.0) for 10 minutes. Electrochemical measurements were performed in Cd^{2+} free 50 mM ammonium acetate and 50 mM NaCl. Voltammograms were measured at a pulse amplitude of 0.025 V, a step of 0.004 V and a frequency of 25 Hz. Reproduced with permission of The Royal Society of Chemistry from [28], Copyright, Royal Society of Chemistry (2003).

The generic aspect of the electrode fabrication protocol means that any peptide sequence can be modified at a $-COOH$ functionalized surface in a similar manner. With this approach, oligopeptides and polypeptides have been attached to gold electrodes as biorecognition elements for the determination of copper [30–33], lead [35] and silver [77] as well as cadmium. Perhaps the best demonstration of the power of peptide modified electrodes using this interface is with the copper binding peptide, Gly-Gly-His, by covalent attachment to MPA (Fig. 3c) [31–33]. Copper binding using Gly-Gly-His has been widely studied by pH titrimetry and spectrophotometric methods. It serves as a model for human serum albumin where the binding site for copper is the *N*-terminal Asp-Ala-His which is responsible for transporting copper between tissues and blood. Extraordinarily low detection limits with Gly-Gly-His modified electrodes were reported; sub-ppt detection limits and minimal interference from other metal ions (apart from Ni^{2+}) [31]. These detection limits are very sensitive to the quality of the self-assembled monolayer on the gold electrode surface. The detection limits are so low they cannot be validated independently and the concentrations employed are only nominal concentrations. However, the detection limits are consistent with the known equilibrium constants for the binding of copper by this peptide in solution [32]. The study with Gly-Gly-His modified electrodes does highlight that the use of peptide modified electrodes may provide sensors for free metal ions with unrivalled sensitivity.

5. Challenges and Future Prospects

The application of peptide modified surfaces for metal ion sensing is in its infancy. The recent work by our group and by others has demonstrated there is enormous potential for using peptide modified electrodes for the detection of metal ions and in the sequestration of metals from water samples. With regards to sensing, future prospects exist in screening different peptides for their selectivity for specific metals directly on electrode surfaces, the development of electrode arrays for the detection of multiple metal ions with a single sensor and taking metal ion sensors employing peptides into the nanoscale. There are, however, challenges to overcome to achieve these goals and to make sensors for detecting metal ions in this way commercially viable.

If peptides are to be screened for their selectivity for particular metal ions important challenges to overcome include being able to reliably synthesize peptides directly on electrode surfaces from individual amino acids and to be able to perform such syntheses combinatorially. Reliable synthesis of peptides at an electrode surface will certainly be influenced by the design of the interface to which the individual amino acids will be attached. An important criterion with regards to interfacial design will be providing sufficient space between coupling points on the electrode surface so that peptides can be synthesized without steric hindrance from adjacent ligands. Self-assembled monolayers can provide suitable control over the surface coverage can be made by using a mixed monolayer. Using a mixed SAM of MPA and a diluent 3-mercaptopropane (MP) it has been shown that the tripeptide Gly-Gly-His can be synthesized from individual amino acids to give an electrode with similar copper complexation ability to if presynthesized and purified Gly-Gly-His was attached to the same monolayer [34]. Reliable synthesis of the peptide ligands was confirmed using a specially developed mass spectrometry method [36, 78]. The best electrode performance and highest synthetic yield was obtained with a SAM formed from a 1 : 1 solution of MPA:MP. At lower ratios of MPA:MP there were insufficient ligands formed on the electrode surface whilst at higher surface densities of coupling sites the final synthetic yield was lower (Fig. 5) [34].

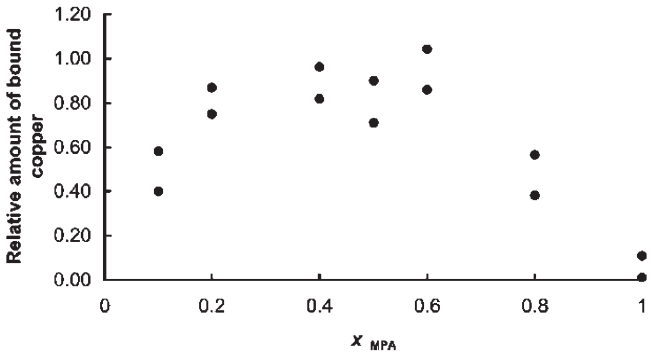


Fig. 5. Relative current due to amount of surface bound copper at a Gly-Gly-His modified electrode versus the mole fraction of MPA in the original monolayer to which the peptide was attached. The relative current is the Cu^{2+} current of Gly-Gly-His synthesized from individual amino acids divided by the Cu^{2+} current when presynthesized Gly-Gly-His was immobilized onto a self-assembled monolayer modified gold electrodes with the same fraction of MPA to MP. Mixed SAMs comprising 3-mercaptopropionic acid and 1-mercaptopropropane were prepared by immersing the gold-coated substrates in solutions of mixtures of 3-mercaptopropionic acid and 1-mercaptopropropane of a given fraction. In each case Cu^{2+} was accumulated at the modified electrode at open circuit for 10 minutes in a 0.05 M ammonium 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 sweep rate was 100 mV/s. Repeat experiments, as represented by two points, are shown at each mole fraction. Reprinted with permission from [36]. Copyright (2005) American Chemical Society.

The highlight of this work is that peptides can be reliably synthesized on an electrode surface one amino acid at a time by simply controlling the surface density of coupling points. Being able to reliably synthesize peptide ligands directly on the electrode surface opens the door to combinatorial synthesis of peptides on electrode arrays for screening metal ion binding ability using either photolithography inspired strategies [79] or microfluidic approaches [80] for example.

The sensors described in this review thus far have focused on the determination of a single analyte using selective ligands. Although these sensors are appropriate for the analysis of a particular metal in a water sample, water samples frequently contain numerous metal ions and it is desirable to determine the concentration of each of these reliably. Sensors employing ligands with a broad selectivity for metal ions may suffer from interfering ions, making accurate quantification of each metal ion difficult whereas highly selective ligands restricts the determination to a single analyte. The solution to this is to use an array of sensors, modified with different ligands, which could provide a range of responses for each analyte. The different patterns of responses may then be deconvoluted using a chemometrics technique [81]. An electrode array for the simultaneous determination of Cu^{2+}, Cd^{2+} and Pb^{2+} [39] which used the peptides Gly-Gly-His, GSH and angiotensin I respectively has recently been described. The three peptides were covalently attached to thioctic acid (TA) SAMs on gold electrodes with a fourth electrode modified with TA only. The main challenges were the overlapping electrochemistry of the Cd^{2+} and the Pb^{2+} and the potential for the same element of the array binding with more than one metal. The voltammetric current of Cu^{2+}, Cd^{2+} and Pb^{2+} was calibrated using multi-way partial least squares regression and was successfully applied to the analysis of test solutions. The advantage of this four electrode approach is that each peptide ligand does not need to be highly selective for a particular metal and that deconvolution takes into account the cross-interfering effects. This is clear in Figure 6 where there is overlap between the redox peaks for lead and cadmium but yet the least squares regression analysis enables deconvolution of these signals and the reliable estimation of these different metals in the same sample.

The success of peptide modified electrodes for detecting metal ions has partly been based on the immobilization of the peptides using self-assembled monolayers with short alkyl chains. The short alkyl chains of the self-assembling molecules have enabled the metal to be complexed close to the electrode surface, and thus be electrochemically accessible. Despite their many advantages, SAMs do have some limitations. The most problematical of these for short chain alkanethiol SAMs is stability of the monolayer with regards to both long term storage and the limited potential range over which the monolayer will stay adsorbed to gold. This issue restricts the determination of metals to ones that are electroactive within a small potential window (typically plus or minus a few hundred mV). When MPA-angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) was used for sensing (Fig. 3e), stable electrochemistry of Pb^{2+} was not

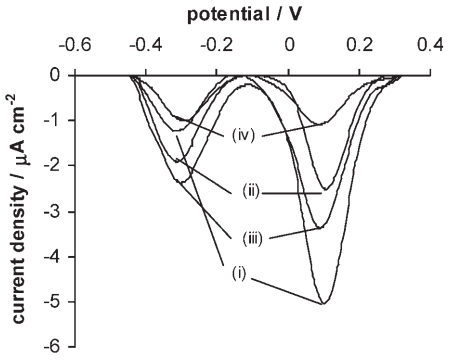


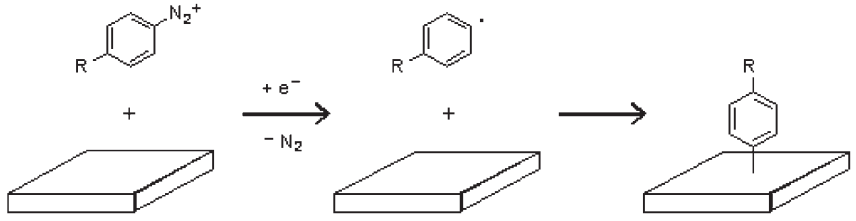
Fig. 6. Baseline subtracted OSW voltammograms of the difference between the voltammograms obtained before and after metal ion accumulation in 0.21 μM Cu^{2+}, 2.4 μM Cd^{2+} and 0.81 μM Pb^{2+} in 50 mM ammonium acetate (pH 7.0) for 10 minutes at 25 °C. The voltammograms are of electrodes modified with i) TA-Gly-Gly-His, ii) TA-glutathione, iii) TA-angiotensin I and iv) TA. OSW voltammograms were measured in 50 mM ammonium acetate (pH 7.0) and 50 mM NaCl at 25 °C at a pulse amplitude of 0.025 V, a step of 0.004 V and frequency of 25 Hz.

possible since the potential occurred just outside the region of stability [35]. An alternative solution was proposed using thioctic acid (TA) SAMs for coupling the peptide. The TA-angiotensin I modified electrode had clear advantages over the MPA-angiotensin I modified electrode due to the wider stability range of the SAM. Cyclic voltammetry of complexed Pb^{2+} at the TA-angiotensin I modified gold electrode showed stable electrochemistry with excellent regeneration and reusability of the modified electrode (less than 1% decrease in the lead current per regeneration cycle) [35]. The compromise, however, was a slight decrease in sensitivity which was attributed to the carboxyl groups of the TA linker being located further away from the electrode surface.

Using SAMs such as TA is not a long-term solution to the stability problem since the potential range for stability is only slightly broadened. Since the stability is affected by the Au-S linkage, an alternative monolayer and or electrode substrate is required for metal determination. One alternative involved the covalent modification of glassy carbon surfaces with aryl diazonium salts via electrochemical reductive adsorption [82, 83]. This has attracted considerable interest recently since aryl diazonium salts can be derivatized with various functionalities and the monolayers formed are highly stable and possess a wide potential range [84]. When the Gly-Gly-His ligand was attached to a benzoic acid modified glassy carbon (Scheme 4) and used for the determination of Cu^{2+}, the sensor was found to be stable for over several months use [37]. In comparison, MPA-Gly-Gly-His modified at a gold surface showed a gradual decline in the response to Cu^{2+} with a loss of ~1% per regeneration when tested once a day [33]. However, there was one drawback of using peptide modified glassy carbon electrodes for sensing and this was the high capacitance of glassy carbon which limited the analytical sensitivity to some extent.

More recent reports have shown that aryl diazonium salts can be successfully grafted on gold surfaces [85, 86] and this has recently been applied to metal ion sensing. Initial experiments have shown that Gly-Gly-His, GSH and angiotensin ligands attached to aryl modified gold surfaces could be used for the determination of Cu^{2+}, Cd^{2+} and Pb^{2+} respectively [38]. Importantly, the monolayers were shown to be far more stable than alkanethiol based monolayers on gold electrode.

Compton and co-workers have also exploited diazonium salt modified graphite electrodes for the sequestration of Cd^{2+}. The high Cd^{2+} binding capacity of poly-L-cysteine is exploited for the removal of Cd^{2+} from water samples by immobilizing the polyamino acid on derivatized graphite powder [24] (Fig. 7). The idea of using polypeptides for the sequestration of metal ions was first reported by Holcombe and co-workers using poly-L-cysteine [87, 88], poly-L-aspartic acid [89, 90], poly-L-histidine [91] and poly-L-glutamic acid [90] physisorbed onto on controlled pore glass which was packed into microcolumns for use in a flow injection analysis system. The important feature was the generic approach to modifying the carbon surface with peptide, especially. The modification of the graphite was achieved using 4-nitrobenzene diazonium to give a nitrophenyl modified graphite. The nitro group was reduced to and amine thus allowing the coupling of peptides from their carboxylic acid terminus. This generic surface chemistry was then applied to the immobilization of poly-L-cysteine. The high surface area of graphite powder allows greater amounts of poly-L-cysteine to be coupled to the surface, thus allowing it to accumulate more Cd^{2+} than if poly-L-cysteine was modified on other solid-state support materials. The uptake of Cd^{2+} by the poly-L-cysteine modified graphite powder was monitored using linear sweep stripping voltammetry at a boron doped diamond electrode after filtering the powder from the test solution. This sensitive electrochemical



Scheme 4. Modification of glassy carbon surfaces via the one electron reduction of aryl diazonium salts.

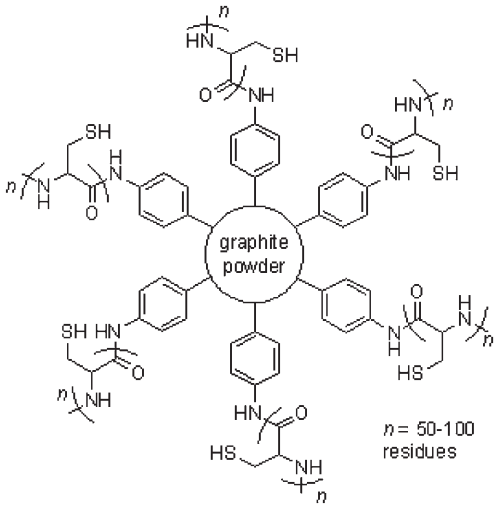


Fig. 7. Graphite powder modified with poly-L-cysteine. The value of *n* for poly-L-cysteine is between 50 and 100 monomer units per polymer chain.

technique detected the amount of Cd^{2+} not chelated by the powder. The uptake of Cd^{2+} was determined to be 137 ± 20 mg Cd^{2+} per gram of poly-L-cysteine modified graphite powder [24] which is up to one hundred more effective than studies in which poly-L-cysteine was immobilized on glassy carbon [23].

The modification of glassy carbon spherical powder with L-cysteine methyl ester has also been an effective material for the removal of Cd(II), Cu(II) and As(III) from aqueous solutions with binding to thiol groups being the dominant mechanism [25, 92].

Extending the use of peptide modified electrodes for the detection of metal ions into the nanoscale is a challenge that is being met by Tao and co-worker [93, 94] who have shown peptide complexing of metals can be used in molecular junctions as a sensing technology. The basic idea is shown in Figure 8 where a gold surface is modified with peptides such as cysteamine-Cys, cysteamine-Gly-Cys, Cys-Gly-Cys or cysteamine-Gly-Gly-Cys; all peptide sequences with thiol moieties at each end. Using a STM tip to form the other contact of a molecular junction, Tao and co-workers have shown using a statistical method that (Figure 8c) electron tunneling through a single molecule can be measured [95]. When these surfaces are exposed to Cu^{2+} or Ni^{2+} and then the conductance measured, the conductance for a single molecular junction increases by up to two orders of magnitude. Furthermore, the change in conductance is different for different ionic species which indicates a single peptide-modified surface can detect several different metals with similar binding configurations. Tao and co-workers suggest the dominant mechanism responsible for this increase in conductivity is the change in conformation of the peptide with electron transfer being mediated by the metal ion. This study was extended by modifying polyaniline with Gly-Gly-His. A nanojunction between two electrodes less than 60 nm apart was formed by electrodeposition of the peptide modified polyanaline [94]. The final sensor

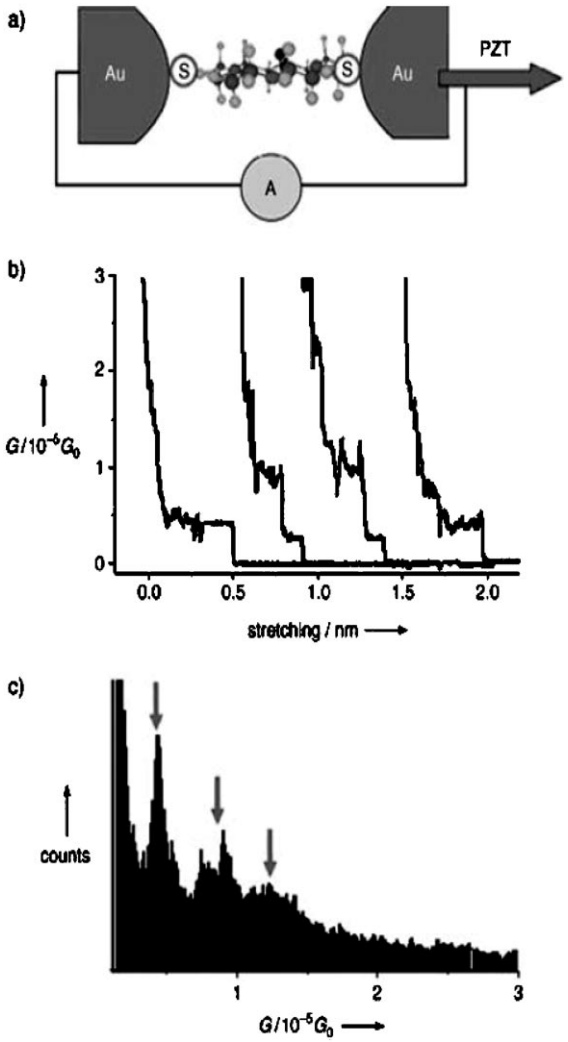


Fig. 8. The application of molecular junctions for detecting metal ions using peptides: a) schematic of a molecular junction formed by the separation of an STM tip (using a piezoelectric transducer - PZT) from a peptide modified surface, b) typical conductance curves of cystamine-Gly-Cys during the stretching of the molecular junctions, and c) conductance histogram constructed from over 500 individual conductance curves. The arrows, from the left, indicate single, two, and three molecule junctions. Reprinted with permission from Wiley-VCH from [93]. Copyright (2004).

was capable of detecting Cu^{2+} or Ni^{2+} in the ppt range. The application of peptides for detecting metals in nanojunctions is particularly exciting as the devices rely on a few or even a single recognition event to give a signal. Work by Lieber and co-workers on silicon nanowires [96, 97] have shown that biosensors which operate on the level of a few binding events can have favorable properties over macroscale devices [98] with regards to sensitivity and selectivity.

The final challenge is to extend the idea of using peptide modified electrodes for detecting non-electroactive ions. With the exception of the ion-gating approach of Takehara et al. [26] the detection of ionic species using peptide

modified electrodes has relied on the natural electroactivity of the metal ion. The ion-gating method of Takehara did however suffer from problems of selectivity. The generic aspects of using peptide modified electrodes for detecting metal ions will be greatly enhanced by being able to detect non-electroactive metals. This is a challenge we are currently attempting to solve.

6. Conclusions

Peptide modified electrodes for the detection of metal ions have produced sensors with unrivalled sensitivity and good selectivity. The power of using peptides is, with generic methods of modifying electrodes, a single technology can be used for the development of a variety of selective metal ion sensors. Such versatility has been demonstrated for the detection of multiple metals on a single electrode array. The initial work that has been performed thus far indicates there is considerable potential for peptide modified electrodes as commercial metal ions sensors but there are still a few hurdles to overcome before this can become a reality.

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