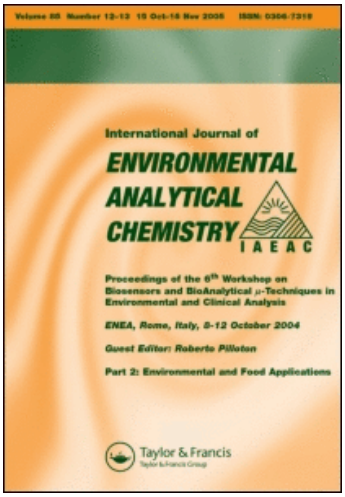

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Comparison of two glutathione S-transferases used in capacitive biosensors for detection of heavy metals

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Comparison of two glutathione *S*-transferases used in capacitive biosensors for detection of heavy metals

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This work describes the development of a heavy-metal biosensor based on either recombinant 6His-Tag glutathione *S*-transferase (GST-$(His)_6$) or glutathione *S*-transferase Theta 2-2 (GST-theta 2-2), and a capacitive transducer. The dynamic range of the pure bovine liver GST-Theta 2-2 biosensor was 1 fM to 1 mM for Zn^{2+}, and 10 pM to 1 mM for Cd^{2+}. The GST-$(His)_6$ biosensor was able to detect Zn^{2+} and Cd^{2+} in the range of 1 fM to 10 μM, and Hg^{2+} in the range of 1 fM to 10 mM. The bovine liver GST Theta 2-2 biosensor displays an increased selectivity and a wider dynamic range for Zn^{2+} compared with the GST-$(His)_6$ biosensor. Therefore, by using different GST isozymes, it is possible to modulate important characteristics of capacitive biosensors for the detection of heavy metals.

Keywords: Capacitive biosensor; 6His-tag glutathione *S*-transferase; Bovine liver glutathione *S*-tranferase theta 2-2; Heavy-metal detection

1. Introduction

Heavy-metal pollution is a threat to human health, animals, and plants. They are not biodegradable and therefore are retained indefinitely in the ecological systems and in the food chain. Therefore, the development of easy-to-use, sensitive, and selective analytical tools for detection of heavy metals is of great importance [1].

Classical analytical methods for detection of heavy metals require time-consuming and labour-intensive sample preparation steps. For example, very often they require the combination of separation steps (such as HPLC, GC, and CE) with complicated detectors (such as mass spectrometry [2], and NMR [3]). Moreover, classical analytical methods (such as Atomic Absorption Spectroscopy (AAS), Atomic Emission

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>sp|P30713|GSTT2_RAT Glutathione S-transferase Theta-2 (EC 2.5.1.18)
(GST class-theta-2) (Glutathione S-transferase 12) (GST 12-12)
(Glutathione S-transferase Yrs-Yrs)-Rattus norvegicus (rat) 27.3 kDa.

GLELYLDLLSQPSRAVYIFAKKNGIPFQLRTVDLLKGQHLSEQFSQVN
                                                *
CLKKVPVLKDGSFVLTESTAILIYLSSKYQVADHWYPADLQARAQVH
                                              *                 *
EYLGWHADNIRGTFGVLLWTKVLGPLIGVQVPEEKVERNRNSMVLAL
        *
QRLEDKFLRDRAFIAGQQVTLADLMSLEELIQPVALGCNLFEGRPQLTA
WRERVEAFLGAELCQEAHNPIMSVLGQAAKKTLPVPPPEAHASMMLRI
                          *                       *
ARIP
```

Figure 1. Representation of rat glutathione *S*-transferase-Theta 2-2 (EC 2.5.1.18) amino acid sequence in FASTA format (16).

Spectroscopy (AES), inductively coupled plasma atomic mass spectrometry (ICP-MS) [4, 5]) are most often confined to the perimeters of specialized laboratories and not appropriate for on-site measurements.

Electrochemical biosensors represent a good alternative for a rapid and simple on-site measurement of bioavailable heavy-metal ions (e.g. Cd, Zn, Cu, Hg, Ni, Fe, Co, Mo, Pb) [6]. An electrochemical biosensor is an integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is retained in direct spatial contact with an electrochemical transduction element [7]. The transducer part of the electrochemical biosensor is translating the chemical changes resulting from the interaction of the target analyte with the biorecognition element into an electrical signal, which can be for example current, potential or capacitance. In addition to their simplicity, electrochemical biosensors (and the instrumentation necessary to read electrochemical signals) can be easily miniaturized and thus made portable.

Electrochemical capacitive biosensors have been successfully applied to detect heavy-metal ions [8]. Capacitive biosensors based on metal-binding proteins, e.g. metallothioneins (SmtA) [9], regulator proteins (MerR) [10], or phytochelatins (EC20) [11], were recently described in the literature. Even though these biosensors were used to detect a variety of heavy-metal ions, they were more selective to Hg^{2+}. This work was therefore focused on finding new biorecognition elements which can specifically detect heavy-metal ions other than Hg^{2+} (such as Zn^{2+} or Cd^{2+}).

Glutathione-*S*-transferases (GSTs) (EC 2.5.1.18) are a group of multifunctional proteins involved in the detoxification of a broad spectrum of xenobiotics [12]. More than 10 GST isozymes have been found in the cytosolic fraction of rat liver.

GST-Theta 2-2 is one of the isozymes found in rat [13] and bovine liver [14]. Studies with rat liver GST-Theta 2-2 showed that this enzyme is a homodimeric protein, with a His rich amino acid sequence [15]. As the complete sequence of the bovine liver GST-Theta 2-2 is not published, the FASTA format of the rat liver GST-Theta 2-2 is given in figure 1 [16]. Purified bovine liver GST-Theta 2-2 was one of the biorecognition elements used in this study.

The other biorecognition element used in this work, namely *Schistosoma japonicum* GST-$(His)_6$ protein [17], similar to mu (μ) type GSTs, contains four cysteine residues, with only the Cys169 residue buried inside the molecule while the other three, Cys85,

```
>1M9B:6(His)-tag glutathione S-transferase 26.5 kDa

MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRN
KKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPK
                                              *
ERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLK

MFEDRLCHKTGDHVTHPDFMLYDALDVVLYMDPMCLDAFPK
          *                                *
LVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPK
    *                                    * * *
(-HHHHHH)
  * * * * * *
```

Figure 2. Representation of recombinant glutathione *S*-transferase-$(His)_6$ amino acid sequence in FASTA format.

Cys138, and Cys178, and additionally Gly212, Gly213, Gly214 [18], and a 6His-tag residue are located on the surface of the protein, as shown in figure 2.

In this article, a natural protein, GST-Theta 2-2, rich in His residues, and a recombinant protein having 6(His)-tag residues are used and compared for the first time to develop capacitive biosensors for the quantitative determination of heavy metals.

2. Experimental

2.1 *Materials*

The recombinant protein GST-$(His)_6$ and GST-Theta 2-2 were dissolved in 0.1 M Tris-tricine buffer, pH 8.6, to a final concentration of 0.04 mg mL^{-1} protein. Thioctic acid, glutaraldehyde (GA), and bovine serum albumin, were purchased from Sigma (St. Louis, MO). 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) was obtained from Fluka AG (Buchs, Switzerland). Gold rods, 99.99%, used as electrode material (Catalogue No. 26,583-7, 3 mm in diameter), and 1-dodecanethiol were from Aldrich Chemicals (Milwaukee, WI). The heavy-metal salts $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $HgCl_2$, and $Cd(NO_3)_2 \cdot 4H_2O$ were all from Merck (Darmstadt, Germany). All reagents were of analytical grade. Unless specified otherwise, solutions were prepared with water obtained from a Milli-Q system, preceded by a reverse-osmosis step, both from Millipore (Bedford, MA).

2.2 *Biosensor development*

Biosensors were prepared by immobilizing the corresponding protein on the gold surface. The proteins GST-$(His)_6$ and GST-Theta 2-2 in phosphate-buffered saline (PBS) (140 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, pH 7.4) in concentrations of 1 mg mL^{-1} protein, were diluted with 0.5 mL of 0.1 M Tris-tricine buffer, pH 8.6, and the resulting solution was ultra-filtered on a microfilter (Amicon, Beverly, MA) with a molecular-weight cutoff of 3000. After ultrafiltration, the protein was recovered from the filter. The protein concentration was adjusted to approximately

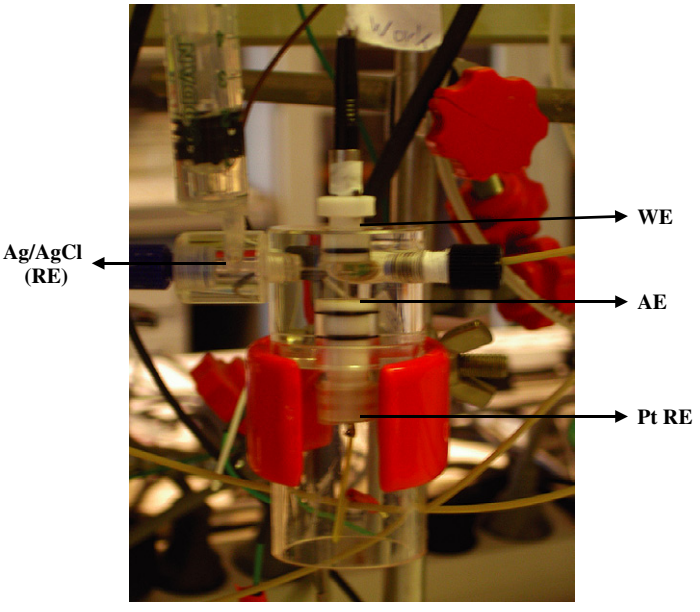

Figure 3. Four-electrode flow-though measuring cell. The electrode system consists of: a gold rod working electrode (WE) mounted in a Teflon holder, a ring-shaped platinum auxiliary electrode (AE), a platinum wire as reference electrode (RE), and an Ag/AgCl electrode, placed on the outlet stream, as a second reference electrode.

$0.04\,mg\,mL^{-1}$ with Tris-tricine buffer to obtain the optimum protein concentration on the gold surface after immobilization.

Gold electrodes were cleaned by polishing with alumina and by plasma cleaning. Subsequently, they were modified with thioctic acid by dipping into 2% thiotic acid in pure ethanol to react overnight at 25°C. Thioctic acid modified electrodes were thoroughly washed with ethanol, dried, and thereafter activated in a 1% solution of EDC in dried acetonitrile for 5 h. After being washed with 100 mM Tris-tricine buffer, pH 8.6, the electrodes were dipped into the protein solution at 4°C for 24 h. As a final step, each electrode was thoroughly washed with Tris-tricine buffer and immersed in a solution of 1-dodecanethiol for 20 min in order to achieve complete insulation of the gold electrode. The successful modification of the electrode was checked by cyclic voltammetry performed in a 5 mM hexacyanoferrate solution containing 0.1 M KCl.

2.3 *Capacitance measurements*

The prepared biosensor was inserted as the working electrode in a specially constructed four-electrode flow cell with a dead volume of 10 µL (figure 3). The electrochemical cell was composed of a working electrode (protein biosensor); platinum wire and platinium foil were the counter and reference electrodes, respectively, and with an extra Ag/AgCl reference electrode placed in the outlet stream. The platinum reference electrode controls the working electrode potential, but it does not have a well-defined potential. Therefore, the platinum and Ag/AgCl reference electrodes were compared

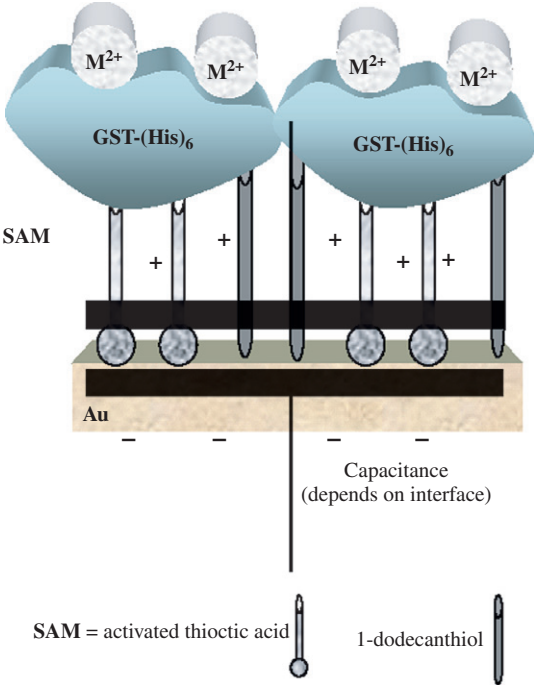

Figure 4. Scheme of protein-modified capacitive biosensor for detection of heavy metals based on conformational change upon binding of the metal ions.

potentiometrically just before the potential step was applied. The computer adjusted the working electrode potential so that the potentiostat behaved as if it had the Ag/AgCl reference controlling the potential on the working electrode.

A potential step of 50 mV was applied for 20 ms (a), and the current response was sampled with a frequency of 50 kHz to evaluate the capacitance change. The heavy-metal-proportional signal, based on the conformational change in the structure of protein which is assumed to take place when the heavy metal ion binds to the protein, was recorded using a fast potentiostat controlled with a computer via a data-acquisition unit [8]. A schematic representation of the detection principle is shown in figure 4. The system buffer solution (10 mM Tris-tricine, pH 8.6), filtered through a 0.22 μm Millipore filter and degassed before use, was pumped by Minipulse 3 peristaltic pump (Gilson Medical Electronics, Villiers-le-Bel, France) with a flow rate of 0.25 mL min^{-1}. $Cd(NO_3)_2$, $ZnCl_2$, and $HgCl_2$ solutions of concentrations ranging from 1 fM to 1 mM were injected into the measuring cell via a 250 μL sample loop.

The capacitance was calculated based on equation (1):

$$i(t) = \frac{u}{R_S} \exp\left(-\frac{t}{R_S C_{tot}}\right), \tag{1}$$

where, $i(t)$ is the current at time t, u is the applied pulse potential, R_s is the resistance of the recognition layer, and C_{tot} is the total capacitance at the electrode/solution interface. The total capacitance of the system is composed by the capacitance of the

self-assembled monolayer (C_{SAM}), the capacitance of the protein (C_P) and the capacitance of the double layer (C_{DL}), described by equation (2):

$$\frac{1}{C_{tot}} = \frac{1}{C_{SAM}} + \frac{1}{C_P} + \frac{1}{C_{DL}}. \quad (2)$$

Each of these capacitances should be made as large as possible in order to detect the capacitance change caused by the biorecognition of heavy metals (C_p).

3. Results and discussion

Proteins can be very effective and often specific recognition molecules for a metal ion [10]. Conformational consequences of metal-ion binding to specific amino acid sequence of the proteins also have a critical impact on the peptide folding processes. Detailed studies on the relations between the amino acid residues and binding capacity revealed a strong binding capacity for heavy metals of Cys [11], Gly, or His [4, 19]. The histidine residue possesses a very efficient electron donor in its side-chain imidazole ring. The cooperation of all three donor groups of this amino acid in metal binding is made possible by the formation of two fused chelate rings [21]. Altun and Köseoğlu (2005) have shown that the π–N of the imidazole ring of histidine is capable of forming stable binary and ternary complexes with Cu(II), Ni(II), and Zn(II) [22]. Moreover, glycine makes complexes with the metal ions via the terminal amine and carbonyl oxygen or amide nitrogen. In a protein with a Gly–Gly sequence, the potential donor atoms are extended to the amide in the peptide backbone. Significantly stronger binding is achieved when the sequence contains several Gly residues in a raw [21]. Therefore, the Gly–Gly–Gly and 6His-tag residues in the GST-$(His)_6$ protein structure, are assumed to take an important role in binding of heavy metals. By using the heavy-metal binding capacity of the GST-$(His)_6$ and GST Theta 2-2 proteins, the affinity of the biosensors were studied for Cd^{2+}, Zn^{2+} and Hg^{2+}. Typical analyte concentration-proportional capacitance changes are shown in figure 5 for GST-$(His)_6$ and in figure 6 for GST-Theta 2-2 biosensors.

Calibration curves obtained for 1 fM to 10 mM Cd^{2+}, Zn^{2+}, and Hg^{2+}, with biosensors using GST-$(His)_6$ and GST-Theta 2-2, are shown in figures 7 and 8, respectively. As shown in figure 7, binding of all three metals to the GST-$(His)_6$ biosensor is observed by capacitance changes which are proportional to the metal concentrations. The pure bovine liver GST-Theta 2-2 biosensor (figure 8) did not exhibit any capacitance change for Hg^{2+} and Cd^{2+} at concentrations lower than 100 nM and 10 pM, respectively. This biosensor appears to be selective for Zn^{2+} in the 1 fM–10 pM range. As the concentration of Zn^{2+} increased from 1 fM to 10 μM, the capacitance change was proportional to concentration change. A further increase in Zn^{2+} and Cd^{2+} concentration considerably increased the sensitivity of the response. The slope of the Cd^{2+} calibration curve is also changed at the above concentration (10 μM).

Figure 9 shows a perfect reproducibility of the GST-$(His)_6$ electrode, prepared, stored, and tested under the same conditions: injection of 250 μL Cd^{2+} after regeneration with 20 mM EDTA, in a 0.1 M Tris-tricine buffer pH 8.6, at a flow rate

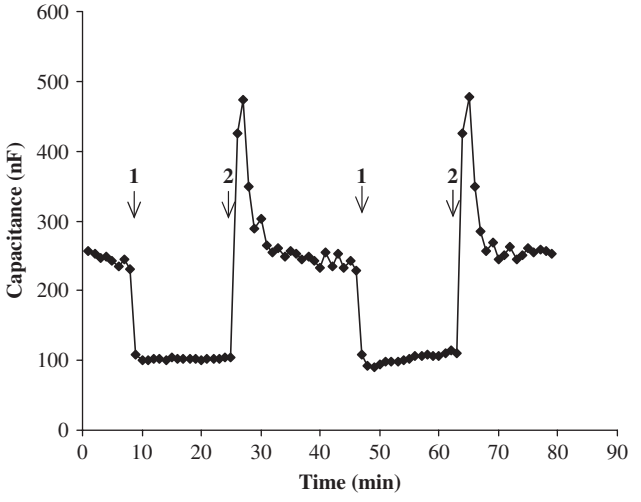

Figure 5. Capacitance changes recorded with the GST-$(His)_6$-modified electrode following injection of 100 μM Cd^{2+} standard solution (1), and 20 mM EDTA regeneration buffer (2). Experimental conditions: 10 mM Tris-tricine buffer, pH 8.6, flow rate of 0.25 mL min^{-1}, injection volume of 250 μL.

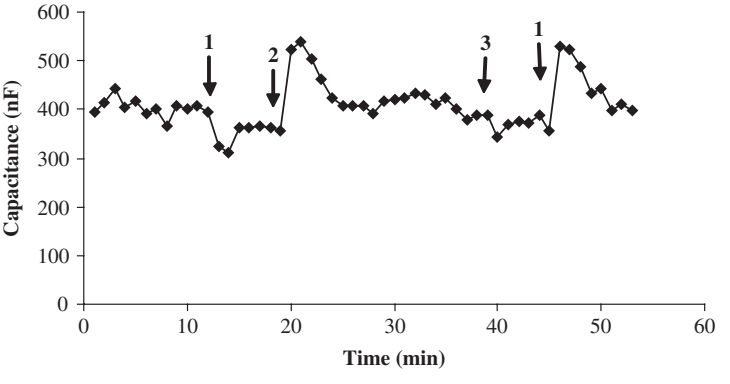

Figure 6. Capacitance changes recorded with the bovine liver GST-Theta 2-2-modified electrode following injection of 1 mM Cd^{2+} standard solution (1), 20 mM EDTA regeneration buffer (2), and 100 μM Cd^{2+} standard solutions (3). Experimental conditions: 10 mM Tris-tricine buffer, pH 8.6, flow rate of 0.25 mL min^{-1}, injection volume of 250 μL.

of 0.25 mL min^{-1}. Reproducibility was also achieved by the measurement of a standard solution over a period of time by the same electrode. The storage stability of the GST-$(His)_6$ and GST-Theta 2-2 biosensors was studied with the injection of 100 μM Cd^{2+} following regeneration with 20 mM EDTA. The biosensors were stored at 4°C in 0.1 mM Tris-tricine buffer, pH 8.6, between measurements. The GST-$(His)_6$ sensor was stable for 14 days, while the GST-Theta 2-2 biosensor was stable for 5 days.

The limit of detection of the GST-$(His)_6$ biosensor is 1 fM to 1 mM for $Zn^{2+} > Cd^{2+} > Hg^{2+}$. The slope of the calibration curve changed after 10 pM and remained constant up to 100 μM Cd^{2+} concentrations. The calibration curve for Zn^{2+} was linearly proportional to Zn ion concentration from 1 fM to 1 mM.

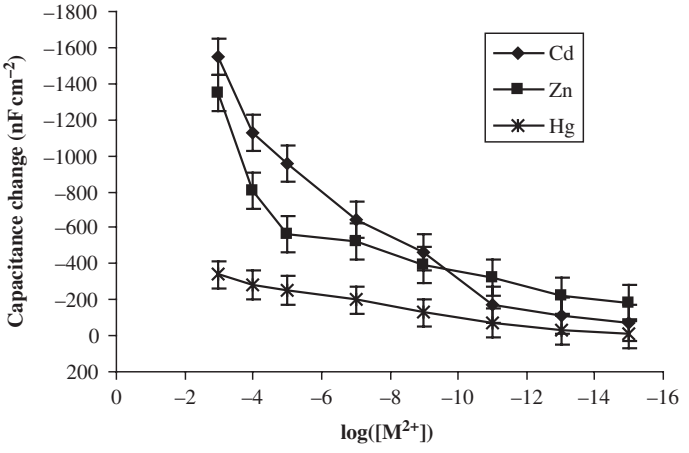

Figure 7. Calibration curves obtained for Cd^{2+}, Zn^{2+}, and Hg^{2+}, with the GST-$(His)^6$-modified electrode. Experimental conditions: 10 mM Tris-tricine buffer, pH 8.6, flow rate of 0.25 mL min^{-1}, injections of 250 µL standard solution.

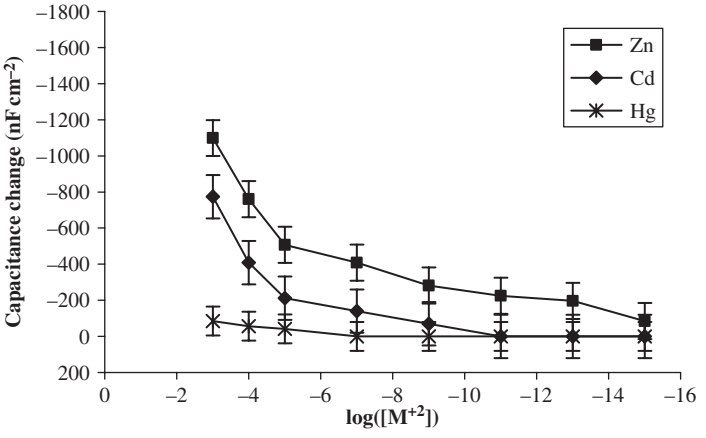

Figure 8. Calibration curves obtained for Cd^{2+}, Zn^{2+}, and Hg^{2+}, with the purified bovine liver GST-Theta 2-2-modified electrode. Experimental conditions: 10 mM Tris-tricine buffer, pH 8.6, flow rate of 0.25 mL min^{-1}, injections of 250 µL standard solution.

The calibration curve for Hg^{2+} was linearly proportional to mercury concentrations greater than 1 pM.

The GST-Theta 2-2 biosensor has a limit of detection as 1 fM to 1 mM for Zn^{2+}, 10 pM to 1 mM for Cd^{2+} and 100 nM to 1 mM for Hg^{2+}. As in the GST-$(His)_6$ biosensor, the calibration curve for Zn^{2+} was linearly proportional to Zn ion concentration from 1 fM to 10 µM. The calibration curve for Cd^{2+} was linearly proportional to Cd ion concentration from 10 pM to 100 mM. The slope of the calibration curve changed after 100 mM to 1 mM Cd ion concentration. The calibration curve for Hg^{2+} was linearly proportional to Hg ion concentration from 100 nM to 1 mM with no detection at low concentrations.

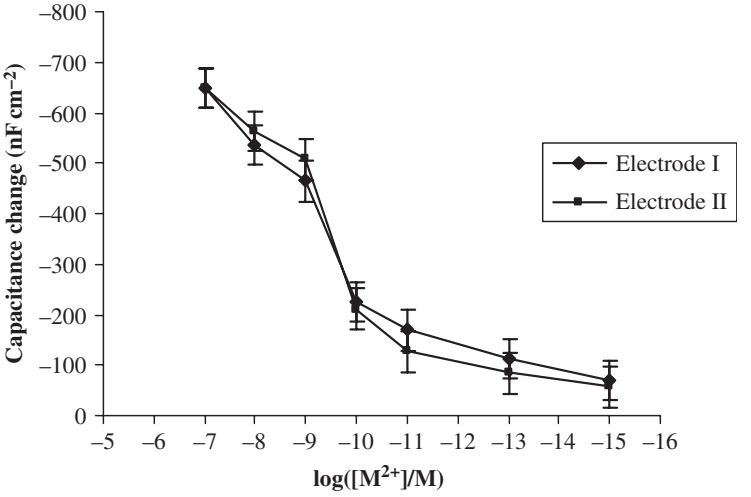

Figure 9. Calibration curves obtained for Cd^{2+} with two GST-$(His)_6$-modified electrodes, prepared at different times. Experimental conditions: 10 mM Tris-tricine buffer, pH 8.6, flow rate of 0.25 mL min^{-1}, injections of 250 μL standard solution.

4. Conclusion

The bovine liver GST-Theta 2-2 biosensor is more sensitive to Zn ions than Cd ions, whereas the GST-$(His)_6$ biosensor shows higher response to Cd ions than that of the bovine liver GST-Theta 2-2 biosensor. Therefore, the bovine liver GST-Theta 2-2 biosensor seems more selective to Zn ions than the GST-$(His)_6$ biosensor. In addition, the bovine liver GST-Theta 2-2 biosensor shows a very small sensitivity to Hg^{2+}, in comparison with the GST-$(His)_6$ biosensor. The observed pattern of biosensors responses could be explained by the differences in amino acid composition between the proteins used in the construction of biosensors. Hence, by using different GST isozymes or by modification of GST isozymes, it is possible to obtain metal specific biocomponents for capacitive biosensors for the detection of heavy metals. Future work targets modifying the proteins and testing of optimal electrode for detection of one metal ion in mixture of metal ions.

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