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Novel synthetic phytochelatin-based capacitive biosensor for heavy metal ion detection

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Abstract

A novel capacitance biosensor based on synthetic phytochelatins for sensitive detection of heavy metals is described. Synthetic phytochelatin $(Glu\text{-}Cys)_{20}Gly$ (EC20) fused to the maltose binding domain protein was expressed in *Escherichia coli* and purified for construction of the biosensor. The new biosensor was able to detect Hg^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+} ions in concentration range of 100 fM–10 mM, and the order of sensitivity was $S_{Zn} > S_{Cu} > S_{Hg} \gg S_{Cd} \cong S_{Pb}$. The biological sensing element of the sensor could be regenerated using EDTA and the storage stability of the biosensor was 15 days.

Keywords: Biosensors; Heavy metals; Phytochelatins; Capacitance

1. Introduction

Evolution of our society has lead to increasing levels of diverse pollutants. Heavy metals represent some of the most toxic ones not only for the environment, but also in many other areas like medicine and food industry. Society is learning to adapt to pollution by heavy metals, and is trying to remediate, control and minimize such pollution as much as possible. In order to achieve this, there is an acute need to develop new methods of assessing the heavy metal concentrations in natural and industrial environments.

There are several methods for heavy metal detection including spectroscopical (AAS, AES, ICP-MS, etc.; Burlingame et al., 1996; Jackson and Chen, 1996) or electrochemical (ISE, polarography, etc.; Anderson et al., 1996) ones. These methods, however, are either expensive or not useful when there is a need to detect metals at low concentrations. Moreover, these methods can only detect the total amount of heavy metals and not the bioavailable concentrations accessible to the living organisms. Therefore, development of new and inexpensive methods for detection of bioavailable heavy metal concentrations is highly desirable.

Biosensors are useful analytical devices in this respect, and several configurations were described in the past for heavy metal detection. There is a great variety in both biorecognition elements and transducers used for biosensor (Bontidean et al., in press). Whole cells (e.g. bacteria, fungi, lichens, mosses (Wittman et al., 1997), enzymes (Budnikov et al., 1991; Zhyliak et al., 1995; Fennouh et al., 1998; Thompson et al., 1998), apoenzymes (Mattiasson and Nilsson, 1979; Satoh and Kasahara, 1990; Satoh, 1991, 1992), antibodies (Khosraviani et al., 1998) or proteins (Bontidean et al., 1998, 2000a,b, 2002) were used as recognition elements coupled to different transducer types, e.g. potentiometric, amperometric, optic, conductometric, spectrophotometric, etc. The main disadvantage of whole cell and enzyme based biosensors consists in their limited selectivity and rather low sensitivity. Biosensors based on metal binding proteins, e.g. metallothioneins (SmtA) or regulator proteins (MerR), coupled to a highly

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sensitive capacitive transducer were recently described in the literature to sensitively detect heavy metals (Bontidean et al., 1998, 2000a,b, 2002). However, these biosensors also detected most of the heavy metal ions, although the Mer-based electrodes were more selective towards Hg^{2+} than the SmtA-based ones. Our attention, has been therefore, focused on finding new biorecognition elements which might detect the bioavailable heavy metal ions individually.

Phytochelatins (PCs) are short, cysteine-rich peptides with the general structure $(\gamma\text{Glu-Cys})_n$ Gly ($n = 2$–11; Fig. 1). PCs offer many advantages over MTs due to their unique structural characteristics, particularly the continuously repeating γGlu-Cys units. For example, PCs have higher metal-binding capacity (on a per cysteine basis) than MTs (Mehra and Mulchandani, 1995). In addition, PCs can incorporate high levels of inorganic sulfide that results in tremendous increases in the Cd^{2+}-binding capacity of these peptides (Mehra et al., 1994). It is, therefore, necessary to investigate PC as an alternative biosensing element to metallothionine for detection of heavy metals.

Isolation and purification of PC from plants or organisms is tedious and time consuming. Production of PC using the recombinant DNA technology is currently not possible because of the insufficient understanding of the enzymes involved in the synthesis and chain elongation of these peptides. Synthetic phytochelatins (ECs) are analogs of PCs that have an α-peptide bond instead of the γ-peptide bond (Fig. 1 A and B). Unlike PCs, ECS can be produced using the cell

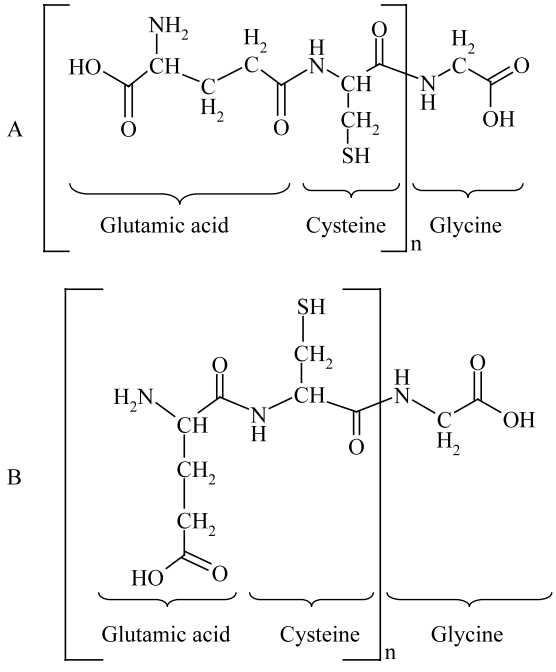

Fig. 1. Chemical structure of natural (PC) and synthetic (EC) phytochelatins.

ribosomal machinery. Additionally, ECS of different chain lengths, even higher than 11 that are found in natural plants, can be produced to provide peptides with different metal binding capacities. Using synthetic genes we have recently synthesized ECs of different chain lengths for removal of heavy metals (Bae et al., 2000).

In this paper, we report on the development, characterization and evaluation of an EC20-based capacitance biosensor for quantitative determination of heavy metals.

2. Experimental section

2.1. Chemicals

Purified EC20 was supplied in buffer with 20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, and >10 mM maltose. Before use, the protein was treated with 5 mM DTT by incubating at 37 °C for 2 h to reduce the disulfide bonds formed during storage of the protein, ultracentrifuged on a filter with a 3000 MWCO Centricon filter (Millipore, Bedford, MA, USA), and the storage buffer was exchanged to 100 mM borate buffer pH 8.75 used as coupling buffer. Gold rods, 99.99%, used as electrode material (Cat. No 26,583–7, 3 mm in diameter) and 1-dodecanethiol were from Aldrich Chemicals, Milwaukee, WI, USA. Thioctic acid was purchased from Sigma, St. Louis, MO, USA, dithiothreitol (DTT) from ICN, Costa Mesa, CA, USA, and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) from Fluka AG, Buchs, Switzerland. The heavy metal salts $CuCl_2 \cdot 2H_2O$, $ZnCl_2$, $HgCl_2$ and $Cd(NO_3)_2 \cdot 4H_2O$ and ethylene-diamino-tetraacetic acid (EDTA) were from Merck, Darmstadt, Germany and $Pb(NO_3)_2$ from Acrōs Organics, Geel, Belgium. All reagents were of analytical grade. Solutions were, if not otherwise specified, prepared with water obtained from a Milli-Q system, preceeded by a reverse osmosis step, both from Millipore, Bedford, MA, USA. To remove heavy metal traces all glassware was soaked in 3 M HNO_3 for 3 days followed by soaking in Millipore water, before use.

2.2. Methods

2.2.1. Protein production and purification

To express EC20 intracellularly, plasmid pMC20 (Bae et al., 2000), which allows the cytoplasmic expression of EC20 as a fusion to the maltose-binding protein (MBP), was used. *Escherichia coli* strain JM105 carrying pMC20 was grown in low-phosphate MJS medium supplemented with 50 μg/ml ampicillin at 30 °C to an OD_{600} of 0.3 when 1 mM IPTG was added to induce the expression of the fusion protein. MBP-EC20 fusion protein was purified from cultures using an amylose resin affinity

column (New England BioLabs, Inc., Beverly, MA, USA). The purity of the protein was confirmed through SDS-PAGE (12.5% (w/v) polyacrylamide).

2.2.2. Biosensor construction

A detailed description of the protein immobilization method has been previously published (Bontidean et al., 1998). Briefly, gold electrodes were thoroughly cleaned (by polishing, sonication and plasma) and pretreated with thioctic acid that formed a self-assembled monolayer (Berggren and Johansson, 1997). The proteins were coupled to the self-assembled monolayer via EDC coupling method. Finally, the electrodes were immersed in a solution of 1-dodecanethiol for 20 min just before inserting them as the working electrode of a three/four electrode electrochemical cell, integrated into a flow injection line.

2.2.3. Capacitance measurements

The biosensor was placed in a specially designed three(four)-electrode electrochemical flow cell. Pt foil and Pt wire served as auxiliary and reference electrodes, respectively. An extra reference electrode (Ag/AgCl) was placed in the outlet stream (Berggren and Johansson, 1997). The electrodes were connected to a fast potentiostat via a Keithley data acquisition and control unit (see Fig. 2).

The 10 mM borate buffer solution used as carrier, was filtered through 0.22 μm Millipore filter, degassed and pumped by a peristaltic pump (Alitea, Gothenburg, Sweden), with a flow rate of 0.25 ml/min. 250 μl samples were injected into the carrier flow via a sample loop. Measurements were made by applying a 50 mV potential pulse and recording the current transients following the potential step. The current values were collected with a frequency of 50 kHz and the first ten values obtained at 1 s intervals were used for the evaluation of the capacitance.

2.2.4. Cyclic voltammetry measurements

Cyclic voltammograms were recorded in a three electrode electrochemical batch cell containing 5 mM $K_3[Fe(CN)_6]$ and 0.1 M KCl, at a scan rate of 100 mV/s. Gold electrodes unmodified or covered by different layers were used as working electrodes, a saturated calomel electrode (SCE) served as reference and a platinum flag as the auxiliary electrode. The electrodes were coupled to a BAS potentiostat (Bioanalytical Systems, Inc., West Lafayette, IN, USA), connected to a computer.

3. Results and discussion

Binding of heavy metal ions to the protein immobilized on the electrode surface will cause a change in protein conformation so that the space-charge in the aqueous solution will move closer to the electrode surface. The conformational change of the protein is measured as a change in capacitance. The total capacitance described by Eq. (1) is composed of a series of capacitances: (i) the capacitance of the self-assembled monolayer of thiol (C_{SAM}), (ii) the capacitance of the protein layer (C_P) and (iii) the capacitance of the double layer (C_{DL}) formed by the solvated ions of the liquid forming a space-charge, as seen from Fig. 3.

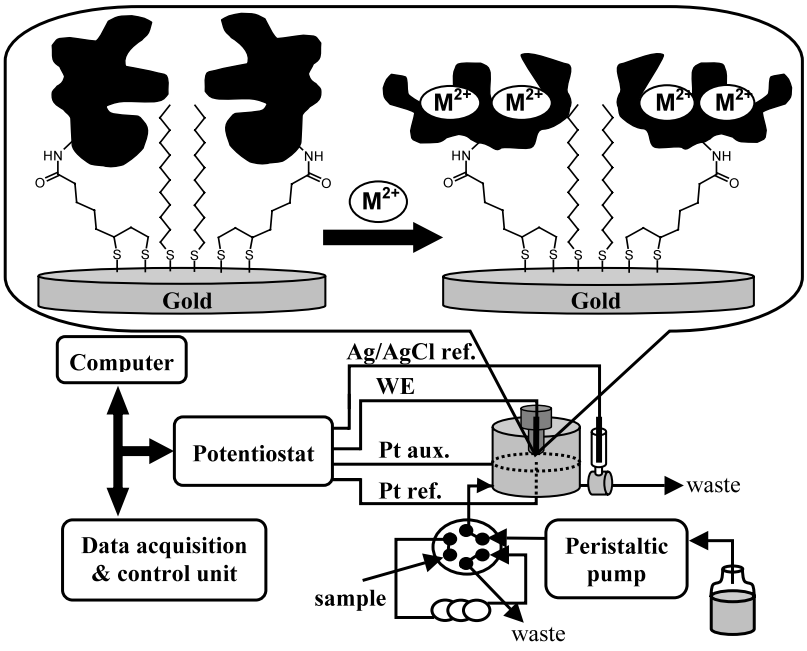

Fig. 2. Schematic drawing of the experimental set-up.

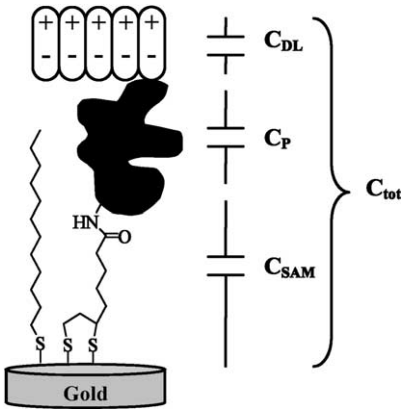

Fig. 3. Schematic drawing of the different layers on the electrode surface determining the total capacitance.

$$\frac{1}{C_{\text{tot}}} = \frac{1}{C_{\text{SAM}}} + \frac{1}{C_{\text{P}}} + \frac{1}{C_{\text{DL}}} \quad (1)$$

The smallest capacitance in this series dominates the value of the total capacitance, therefore, all other capacitances should be as large as possible so that the changes caused by the recognition will dominate.

In case of capacitive measurements a good insulation of the electrode surface is crucial for eliminating unwanted redox reactions and reducing the background current. The degree of insulation obtained at different stages in the electrode preparation is demonstrated by cyclic voltammetry measurements performed in a solution containing a small redox couple such as $K_3[Fe(CN)_6]$ as shown in Fig. 4. On the clean gold surface the reversible peaks for oxidation and reduction of the redox species are observed during cycling of potential. When the self-assembled layer of thioctic acid covers the surface, the redox peaks are considerably reduced because the electrode surface is less accessible for the redox species. Immobilization of protein on the electrode surface increases the insulating properties of the electrode, but only after treating with 1-dodecanethiol for 20 min, the electrodes became totally insulated.

Capacitance measurements were performed by inserting the protein modified electrodes as working electrode in the electrochemical flow cell with three(four) electrodes. The Pt reference controls the potential applied on the working electrode, but it does not have a well-defined potential. Therefore, the potentials of Pt and Ag/AgCl reference electrodes were compared just before the potential pulse was applied and the computer adjusted the potential so that the potentiostat behaved as if the Ag/AgCl reference controlled the working electrode.

The capacitance was calculated according to Eq. (2):

$$i(t) = \frac{u}{R_{\text{S}}} \exp\left(-\frac{t}{R_{\text{S}} C_{\text{tot}}}\right) \quad (2)$$

where $i(t)$ is the current at time t, u the amplitude of the potential pulse applied, R_s the resistance between the gold and the reference electrode, C_{tot} the total capacitance over the immobilized layer, and t is the time elapsed after the potential pulse was applied.

Calibration curves for Hg^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+} and Zn^{2+} were recorded in the 1 fM–10 mM concentration range and are shown in Fig. 5. In all cases heavy metal ions could be detected in a large concentration range starting from 1 fM to 10 mM. The capacitance changes in the low concentration range up to a certain concentration, M* (see Table 1). The M* value depended on

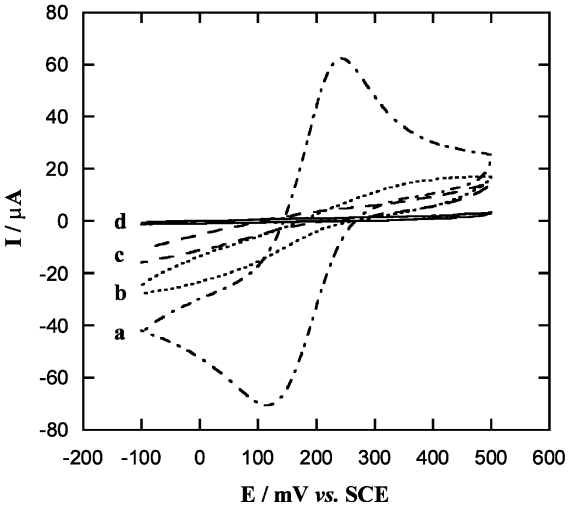

Fig. 4. Cyclic voltammetry measurements for (a) clean gold, (b) thioctic acid covered gold, (c) phytochelatin modified gold, and (d) as in (c) but after 1-dodecanethiol treatment. Voltammograms recorded in 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl, at a scan rate of 100 mV/s.

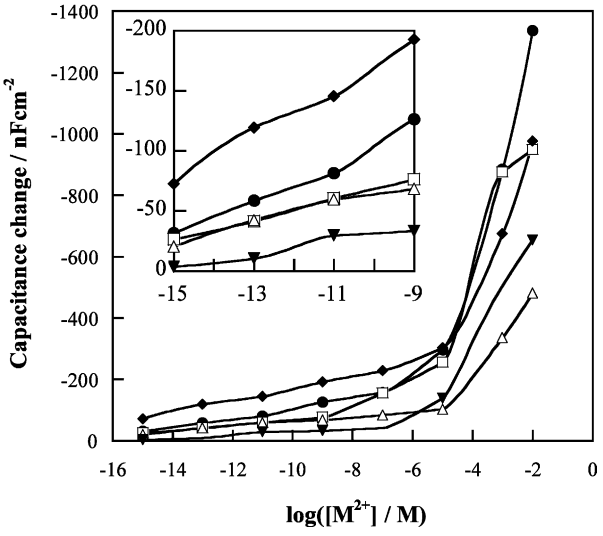

Fig. 5. Calibration curves obtained with EC20 modified electrodes for (●) Cu^{2+}, (◆) Zn^{2+}, (□) Hg^{2+}, (▼) Cd^{2+}, and (△) Pb^{2+}. Measurements were performed in 10 mM borate buffer, pH 8.75, flow rate 0.25 ml/min, and sample injection volume 250 µl.

Table 1
Concentration of heavy metal ions where the slope of the calibration curves changes

Protein	M*				
	Hg^{2+}	Cd^{2+}	Cu^{2+}	Zn^{2+}	Pb^{2+}
EC20	10^{-9}	10^{-7}	10^{-7}	10^{-5}	10^{-5}
SmtA[a]	10^{-9}	10^{-9}	10^{-5}	10^{-10}	10^{-7}
MerR[a]	10^{-7}	10^{-7}	10^{-6}	10^{-7}	

[a] The values for SmtA and MerR were taken from Bontidean et al. (2000a,b).

the type of heavy metal ion. When injecting heavy metal ions in concentrations higher than M*, the slope of the calibration curves changes tremendously. This trend is similar to that observed in our earlier work with other two metal binding proteins, SmtA and MerR (Bontidean et al., 1998, 2000a,b, 2002). Our earlier results with MerR (Bontidean et al., 2000a,b), for which the proof is available, suggests that these capacitance changes may be correlated with the conformational changes occurring in the structure of the protein.

The sensitivity of the EC20-based biosensor for the different heavy metal ions at low concentrations up to 10^{-5} M decreases in the order: $S_{Zn} > S_{Cu} > S_{Hg} > S_{Cd} \cong S_{Pb}$, while above that concentration the biosensor becomes more sensitive to Cu^{2+}. This trend of order of sensitivity is different from that for SmtA and MerR that showed the sensitivity trends of $S_{Cu} > S_{Cd} > S_{Hg} > S_{Zn} \cong S_{Pb}$ and $S_{Hg} > S_{Cu} > S_{Cd} \cong S_{Zn}$, respectively. EC20-based biosensor for all metal ions, except Zn(II), was less sensitive than SmtA- and MerR-based systems. This result is contrary to the expectation that since EC20 has more cysteines per molecule and has higher metal binding capacity, thus, an EC20-based biosensor should be more sensitive. A probable explanation for the lower sensitivity of EC20-based biosensor can be due to lower degree of conformational change induced from metal binding when compared with the case of SmtA. Since the change in capacitance is a function of the resulting conformational change, hence the lower sensitivity for EC20-based biosensor.

In order to assess the influence of the flow rate of the carrier borate buffer on the capacitive signal, calibration curves for Cu(II) were recorded with EC20 modified electrodes at flow rates of 0.25, 0.5 and 1 ml/min. As seen from Fig. 6, the highest signals were obtained for 0.25 ml/min flow rate, which means that at higher flow rates the residence time of the sample solution in the cell is too low, and the metal ions are washed away before they can bind to the protein immobilized on the electrode surface.

An important feature of a biosensor is its reusability. Therefore, regeneration experiments were made by alternative injections of 1 mM EDTA and 100 μM

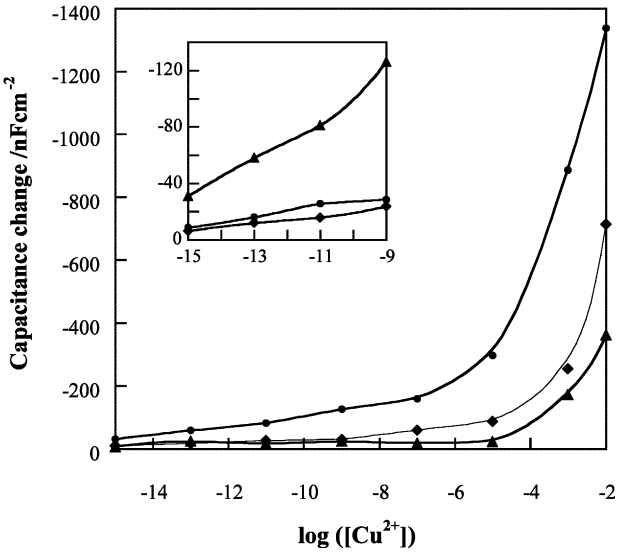

Fig. 6. Calibration curves for Cu^{2+} recorded with EC20 modified electrodes at different flow rates: (●) 0.25, (◆) 0.5, and (▲) 1 ml/min. Experimental conditions are the same as in Fig. 5.

metal ion solution. As an example the regeneration curve for lead is shown in Fig. 7. Regeneration curves for the other metal ions were similar, with the mention that capacitance changes were different for the different ions injected, i.e. 320, 297, 264, 170, and 113 nF/cm^2 for Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}, respectively.

The storage stability of the EC20 modified electrodes was checked during a period of about 3 weeks. A good stability was observed for approximately 2 weeks, after which the signal obtained for injection of 100 mM Zn^{2+} started to decrease. It was also observed that after 15 days, the time needed for the signal to stabilize increased tremendously, from 15 min to between 30 and 60 min, probably due to the fact that in time, after several

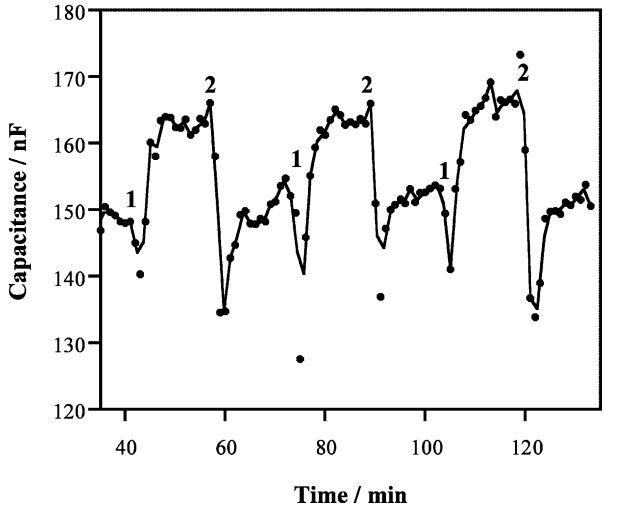

Fig. 7. Regeneration of the EC20 modified electrode with (**1**) 1 mM EDTA after injections of (**2**) 100 μM Zn^{2+}. Other experimental conditions are as in Fig. 5.

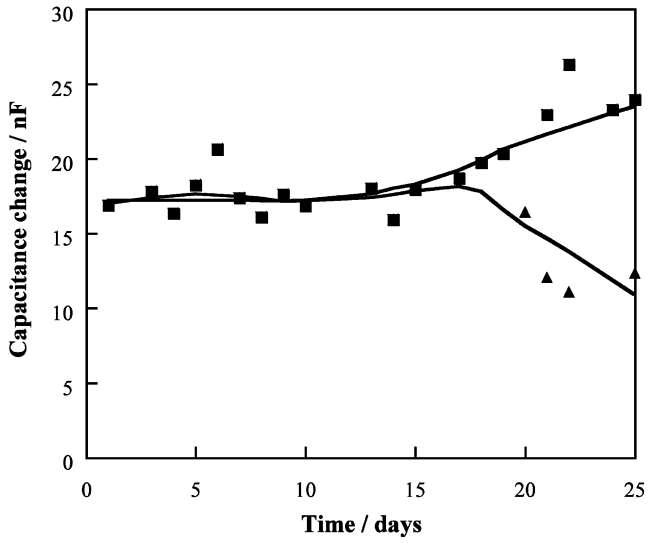

Fig. 8. Storage stability of the EC20 modified sensor. Capacitance changes for 100 μM Zn^{2+} were recorded daily during more than 3 weeks. The capacitance values were measured after 15 (■) and 30 min (▲) from injection of the metal. Between measurements, the sensor was kept at 4 °C in 100 mM borate buffer, pH 8.75.

injection-regeneration cycles the protein chain looses its flexibility. In Fig. 8, the capacitance decrease observed after 15 and 30 min, respectively, are shown. Measurements were performed by injecting 100 mM Zn^{2+} after the electrode was regenerated with 10 mM EDTA. Between measurements, the electrode was stored in 100 mM borate buffer, pH 8.75 containing 0.02% NaN_3, at 4 °C.

4. Conclusions

In conclusion, the present work demonstrated for the first time the possibility of using phytochelatins as biorecognition elements of capacitive biosensors to selectively and sensitively monitor heavy metals ions. Phytochelatin-biosensor has a broad selectivity, sensing all heavy metals, with the best sensitivity for Zn(II). The availability of an additional sensor system based on EC20 along with the SmtA and MerR sensors with different response pattern provides additional data for a potential array sensor capable of detecting and quantifying individual metal ions in a mixture. We are currently, exploring this possibility.

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