The first isoform-selective protein biosensor: a metallothionein potentiometric electrode

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The construction of a biosensor membrane by embedding mammalian Zn_7-MT1 complexes as ionophores in a polysulfone matrix resulted in precise, accurate and, significantly, selective electrodes for MT1 quantification, so that the presence of other mammalian MT isoforms did not interfere in MT1 measurement.

Since their discovery in 1957,[1] metallothioneins (MTs), the ubiquitous, polymorphic metal-binding peptides,[2] have been the subject of wide-ranging multidisciplinary scientific interest.[3,4] The composition, structure and function(s) of the physiologically relevant metal–MT complexes are still matters of debate.[5–7] Concomitantly, the peculiar features and abilities of MTs have triggered many biotechnological applications, mainly aimed at bioremediation and/or biosensor construction. In particular, their metal-binding capacity, together with the inducibility of their encoding genes in response to heavy metals, confers on them a great potential for monitoring metal contamination in health and environment surveillance rationales.[8] As a rule-of-thumb, it is widely accepted that the amount of metal-loaded MT in an organism is a good indicator of its exposure to heavy metals, and thus of the level of contamination of its habitat. However, the accurate quantification of MTs from biological samples still remains a challenge, with a large variety of methodologies, usually suboptimal, to choose from (reviewed in ref. 9). In most cases, quantification of MT protein requires total MT isolation from crude samples (*i.e.* cell cultures, biological fluids or tissues, organism homogenates) followed by MT isoform purification, overall encumbering in practice the use of MTs as efficient and easy to handle biological markers.

MT quantification usually relies on conventional indirect or direct methods coupled to different detection techniques. Indirect methods are based on electroanalytical or spectrometric measurement of the metal ions (usually Ag(I),[10] Hg(II)[11,12] or Cd(II)[13]) bound to an MT after its saturation (*i.e. metal-saturation assays*). However, a precise knowledge of the metal–MT stoichiometry for each MT isoform is essential to apply this method, and this information is not always reliable, available or unanimous.[9] Conversely, direct methods aimed at protein quantification have been developed, mainly by immunological approaches (radioimmuno- (RIA) or enzyme-linked immunosorbent- (ELISA) assays).[14] Nevertheless, accuracy in this case requires suitable standards of the different MTs, not always available, and, significantly, isoform-specific antibodies, which have not been obtained for the vast majority of known MT isoforms. Finally, at gene level, the determination of the levels of MT mRNA in biological samples has also been proposed,[9] on the basis of ion presence in the medium. Latest developments of electrochemical methods yielded the most significant advances in the field. Hence voltammetry, chronopotentiometry and polarography applications have lowered the detection range to as low as 10^{-7} to 10^{-10} M,[9] and recently even zeptomolar sensitivity has been reported.[15] Despite these attractive perspectives, electrochemical approaches entail high preparation purity, due to the interference caused by different compounds in the sample.[16] In this scenario, we consider it to be of noteworthy relevance to communicate the construction and features of the first reported MT isoform-selective sensor, a potentiometric electrode for the direct quantification of mammalian MT1. We show that other MTs, specifically MT4, another mammalian MT isoform, do not interfere in the quantification of MT1, as revealed by the results of calibrations of this new electrode performed in its presence.

The mouse MT1 and MT4 isoforms are homologous, highly similar proteins (Table 1), whose metal binding features have been extensively compared.[17–19] For this study they were recombinantly (*E. coli*) synthesized as Zn_7-MT complexes following a previously reported GST-fusion strategy that, after a thrombin cleavage step, renders notably homogeneous preparations of the MT portion, in 50 mM Tris-HCl buffer.[17,20] MT concentration and Zn-to-protein ratios of the final preparations were assessed by atomic emission spectroscopy (ICP-AES) measurement of the S and Zn content. Also, the molecular mass of the Zn_7-MT species obtained was confirmed by electrospray ionization mass spectrometry (ESI-MS) following already described procedures.[5]

The electrodes designed in this work were of all-solid-state type using a solid internal contact commonly used in our laboratories[21] and for the assembly of these sensors we used polysulfone as matrix. This material allows potentiometric membrane preparations[22] and is hydrophilic enough to contain substances of biological origin such as enzymes,[23] antibodies[24] or, as in this case, MTs. MT1 was embedded into the matrix as Zn_7-MT1, which directly prevented oxidation of

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Table 1 Amino acid sequence of the mouse MT1 and MT4 isoforms, showing their conserved cysteines, which are the residues responsible for the metal ion coordination through thiolate bonds

MT1	MDP-NCSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKGAADKCTCCA
MT4	MDPGECTCMSGGICICGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKGGSDKCSCCP

the protein in contact with air. Hence, an electrode with a membrane containing the Zn_7-MT1 species in one of its ends was constructed following the procedure we recently applied for metal ion-measuring MT-based electrodes[25] (Fig. 1). The membranes were prepared by placing a drop of a solution of 100 mg polysulfone/ml DMF in the tip of the electrode, which was put in contact with an aqueous and fairly diluted (*ca.* 5×10^{-5} M) solution of Zn_7-MT1. This causes the coagulation of the membrane due to a phase inversion process, *i.e.* the substitution of the organic phase by water, in which polysulfone is not soluble. Concomitantly, during the coagulation process, the aqueous phase sweeps away the Zn_7-MT1 complexes so that they become trapped in the polysulfone matrix. By these means, the Zn_7-MT complexes go directly from the aqueous phase to the membrane, without any contact with organic solvents that could alter them.

The ability of the obtained electrodes to quantify MT1 protein was mainly characterized through potentiometric measurements on multiple calibration curves in the 5.0×10^{-8} to 5.0×10^{-6} M Zn_7-MT1 range. All determinations were performed at least in triplicate, which ensures their reproducibility and statistical accuracy. Measurements were achieved by adding different volumes of a standard Zn_7-MT1 solution to a fixed volume of MiliQ water (mixed solutions method),[25] and the corresponding data were plotted as potential *vs.* logarithm of the MT1 concentration (Fig. 2). The limit of detection was calculated as the MT1 concentration at the intersection point of the extrapolated linear segments of the calibration plots. The results of these measurements, which could be performed within a few seconds, showed good sensitivities (53 mV/dec), extremely low detection limits (1.0×10^{-7} M MT concentration) and linear ranges for MT1 concentrations above 10^{-6} M. Long lifetimes could also be ensured for dry-stored electrodes at 4 °C and in an open atmosphere after the observation that their periodic use for one month did not lead to significant changes in their performance, as occurred when they were used for metal ion measurements.[25] The functional pH range of these biosensors was established to be between pH 2.0 and pH 7.0, by determining their response to the presence of Zn_7-MT1 in solutions of different pH (Fig. 3), adjusted by appropriate additions of nitric acid or sodium hydroxide. Taking into account that pK_a values for mammalian Zn-MTs have been determined to be around 5.0,[26] these results reveal that the method also works for partially metalated and apo-MTs, which according to some authors[27] are emerging as major players in MTs speciation.

Last but significantly not least, and because the main drawback of electrochemical methods is the response to the primary analyte in presence of other electroactive compounds, we also evaluated the possible interferences between MT1 and other proteins. The selectivity of the electrodes, *i.e.* the capacity of discrimination between the analyte and the interferences, has been studied by the Fix Interference Method (FIM),[28] a methodology that implies carrying out potentiometric measurements on solutions prepared with a fixed concentration of the interfering proteins and by varying the analyte (here MT1) concentrations. To this end, we have repeated the previous calibrations in the presence of a 10^{-6} M solution of the MT4 isoform (Table 1), which has been taken as a model for interfering molecules in view of its similarity to other mammalian MT isoforms, and the fact that it could represent a real interference in biological samples. Analysis of the results revealed that there is a clear selectivity of MT1 *vs.* MT4, as for MT1 concentrations higher than 3×10^{-6} M, the presence of background 10^{-6} M MT4 did not affect the corresponding measurement (*cf.* practical coincidence of the two curves in Fig. 2).

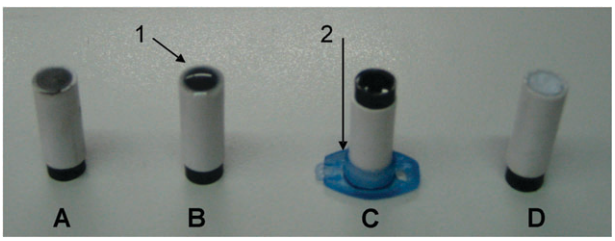

Fig. 1 Preparation process of the polysulfone membranes in the MT selective electrodes. (A) Body of the electrode with the conductive surface. (B) Addition of the solution of polysulfone in DMF; (1) drop of polysulfone in DMF. (C) Immersion of the electrode in the ionophore solution; (2) the blue container encloses the Zn_7-MT1 solution. (D) Polysulfone membrane deposited on the end of the electrode.

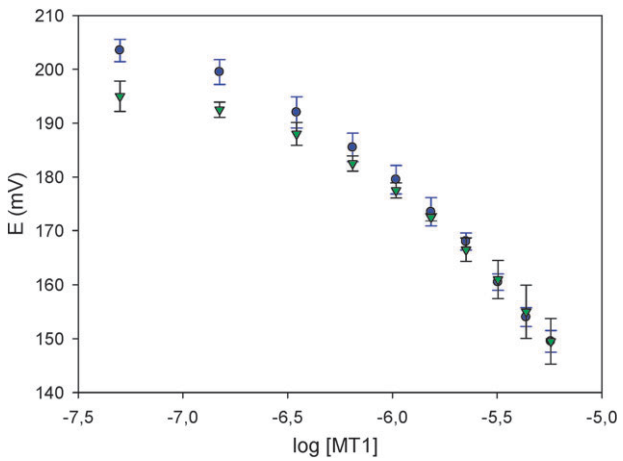

Fig. 2 Calibration of the MT1 selective electrode in water (circles) and in an aqueous solution of MT4 10^{-6} M (triangles). Bars indicate the standard deviation.

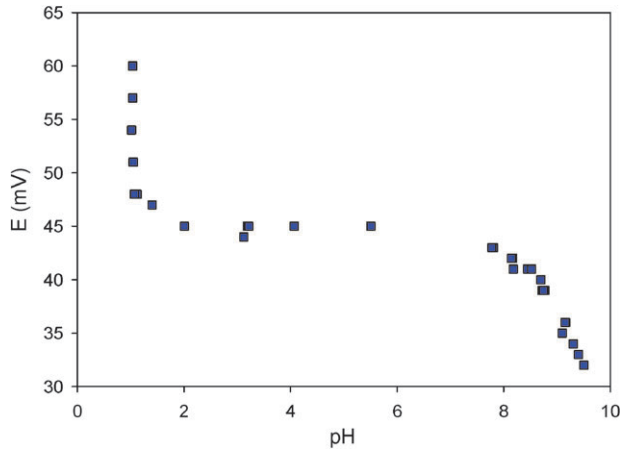

Fig. 3 Effect of the pH on the potentiometric response of the MT-selective electrode, tested at 4.0×10^{-6} M MT1 concentration.

It is true that several MTs, and other metal-interacting polypeptides, have been extensively used for the construction of biosensors, but all of them are capacitance sensors, requiring a regeneration process after each use[29–31] and, most particularly, they are all metal-ion-sensors that make use of the coordinating features of the proteins. However, in no case has a protein-sensor been reported. Thus, to our knowledge, no previous report on the feasibility of using a protein (in this case a metal–MT complex) as its own ionophore is available. Furthermore, this and the Ag-ISE previously reported[25] would form a new type of sensors whose response mechanism could be close to that described for ion-exchange resin membranes but differing in the fact that the interaction between the ionophore and the analyte responds to a complexation process, which confers on them a higher selectivity depending on the different affinities of the analyte and the interferent for the ionophore. In our case, the fact that MT4 is less effective than MT1 for Zn(II) coordination[17] provides a reasoning for the observed selectivity of the MT1-ISE against MT4.

Therefore, the new analytical device here presented, based in ISEs, where the ion is the MT protein, opens new perspectives for developing useful and straightforward MT determination rationales. It shows a patent selectivity and requires neither incubation nor regeneration processes. It is inexpensive because of the small amounts of protein required, it can be dry-stored in an open atmosphere, and is robust and has long lifetimes. Furthermore, its speed of response, simplicity and flexibility together with its portability make this sensor convenient for automated methodologies that can be applied for in-line analysis. Finally, if miniaturization processes are considered for these electrodes, attractive applications concerning direct measurements in tissues or cells are immediately suggested.

Overall data presented here support the hypothesis that small proteins or metalloproteins can be used in polysulfone matrixes as ionophores and open up a broad range of new possibilities in the use of MTs, and perhaps other proteins, for potentiometric sensors.

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Notes and references

1 M. Margoshes and B. Vallee, *J. Am. Chem. Soc.*, 1957, **79**, 4813.
2 http://www.unizh.ch/~mtpage/classif.html.
3 *Metal ions in Life Sciences, Vol. 5: Metallothioneins and Related Chelators*, ed. A. Sigel, H. Sigel and R. K. O. Sigel, Royal Society of Chemistry, Cambridge, 2009, pp. 1–514.
4 P. Gonzalez-Duarte, in *Comprehensive Coordination Chemistry II, Vol. 8*, ed. J. A. McCleverty and T. J. Meyer, Elsevier-Pergamon, Amsterdam, 2003, pp. 213–228.
5 M. Capdevila, J. Domènech, A. Pagani, L. Tío, L. Villarreal and S. Atrian, *Angew. Chem., Int. Ed.*, 2005, **44**, 4618.
6 R. Palmiter, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 8428.
7 P. Coyle, J. C. Philcox, L. C. Carey and A. M. Rofe, *Cell. Mol. Life Sci.*, 2002, **59**, 627.
8 J. C. Gutierrez, F. Amaro and A. Martin-Gonzalez, *BioEssays*, 2009, **31**, 805, and references therein.
9 M. Dabrio, A. R. Rodriguez, G. Bordin, M. J. Bebianno, M. De Ley, I. Sestáková, M. Vasák and M. Nordberg, *J. Inorg. Biochem.*, 2002, **88**, 123, and references therein.
10 J. del Ramo, A. Torreblanca, M. Martínez, A. Pastor and J. Díaz-Mayans, *Mar. Environ. Res.*, 1995, **39**, 121.
11 J. F. Klaverkamp, K. Wautier and C. L. Baron, *Aquat. Toxicol.*, 2000, **50**, 13.
12 P. Shaw-Allen, M. Elliot and C. H. Jagoe, *Environ. Toxicol. Chem.*, 2003, **22**, 2005.
13 J. P. Valles Mota, A. R. Linde Arias, M. R. Fernández de la Campa, J. I. García Alonso and A. Sanz-Medel, *Anal. Biochem.*, 2000, **282**, 194, and references therein.
14 M. M. Chu, Z. Q. Guo, N. Muto, N. Itoh, K. Tanaka and H. W. Ren, *Front. Biosci.*, 2006, **11**, 2113.
15 V. Adam, J. Baloun, I. Fabrik, L. Trnkova and R. Kizek, *Sensors*, 2008, **8**, 2293.
16 J. Petrlova, S. Krizkova, O. Zitka, J. Hubalek, R. Prusa, V. Adam, J. Wang, M. Beklova, B. Sures and R. Kizek, *Sens. Actuators, B*, 2007, **127**, 112.
17 L. Tío, L. Villarreal, S. Atrian and M. Capdevila, *J. Biol. Chem.*, 2004, **279**, 24403.
18 B. Cai, Q. Zheng and Z-X. Huang, *Protein J.*, 2005, **24**, 327.
19 G. Meloni, K. Zovo, J. Kazantseva, P. Palumaa and M. Vasak, *J. Biol. Chem.*, 2006, **281**, 14588.
20 N. Cols, N. Romero-Isart, M. Capdevila, B. Oliva, P. Gonzàlez-Duarte, R. Gonzàlez-Duarte and S. Atrian, *J. Inorg. Biochem.*, 1997, **68**, 157.
21 S. Alegret and E. Martínez-Fàbregas, *Biosensors*, 1989, **4**, 287.
22 A. González-Bellavista, J. Macanás, M. Muñoz and E. Fàbregas, *Sens. Actuators, B*, 2006, **115**, 691.
23 B. Prieto-Simon and E. Fàbregas, *Biosens. Bioelectron.*, 2006, **22**, 131.
24 S. Sanchez, M. Roldan, S. Perez and E. Fabregas, *Anal. Chem.*, 2008, **80**, 6508.
25 A. González-Bellavista, S. Atrian, M. Muñoz, M. Capdevila and E. Fabregas, *Talanta*, 2009, **77**, 1528.
26 L.-J. Jiang, M. Vasak, B. L. Vallee and W. Maret, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 2503.
27 Y. Yang, W. Maret and B. Vallee, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 5556.
28 R. P. Buck and E. Lindner, *Pure Appl. Chem.*, 1994, **66**, 2527.
29 I. Bontidean, J. Ahlqvist, A. Mulchandani, W. Chen, W. Bae, R. K. Mehra, A. Mortari and E. Csöregi, *Biosens. Bioelectron.*, 2003, **18**, 547.
30 I. Bontidean, C. Berggren, G. Johansson, E. Csoregi, B. Mattiasson, J. R. Lloyd, K. J. Kenneth, J. Jakeman and N. L. Brown, *Anal. Chem.*, 1998, **70**, 4162.
31 P. Corbisier, C. Van der Lelie, B. Borremans, A. Provoost, V. De Lorenzo, N. L. Brown, J. R. Lloyd, J. L. Hobman, E. Csoregi, G. Johansson and B. Mattiasson, *Anal. Chim. Acta*, 1999, **387**, 235.

