A novel bio-electronic tongue using different cellobiose dehydrogenases to resolve mixtures of various sugars and interfering analytes

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Abstract

A novel application of cellobiose dehydrogenase enzyme (CDH) as sensing element for an Electronic Tongue (ET) system has been tested. In this work CDH from various fungi, which exhibit different substrate specificities were used to discriminate between lactose and glucose in presence of the interfering matrix compound Ca^{2+} in various mixtures. This work exploits the advantage of an ET system with practically zero pre-treatment of samples and operation at low voltages in a direct electron transfer mode. The Artificial Neural Network (ANN) used in the ET system to interpret the voltammetric data was able to give a good prediction of the concentrations of the analytes considered. The correlation coefficients were high, especially for lactose ($R^2 = 0.975$) and Ca^{2+} ($R^2 = 0.945$). This ET application has a high potential especially for the food and dairy industry and also, in a future miniaturized system, for in situ food analysis.

Keywords : bioelectronic tongue; voltammetry; artificial neural networks; cellobiose dehydrogenase; glucose; lactose; calcium

1. Introduction

Electronic tongues (ETs) constitute a relatively new approach to solve problems in analytical chemistry. Even today it is difficult to find recognition elements for cheap and highly selective sensors or biosensors. Here ETs can come to our help. ETs are multi-sensor systems with cross-response that can process the signal using advanced mathematical methods based on pattern recognition and/or multivariate data analysis. These characteristics of the ET system are an advantage due to the possibility to make further interpretation of complex compositions of analytes, to solve mixtures, to differentiate primary species from interfering components or even to distinguish between false responses and true ones (del Valle 2010). ETs can be exploited to quantify a wide variety of compounds in different fields as food and beverage analysis (Cetó et al. 2013a), environment (Nunez et al. 2013; Raud and Kikas 2013) and medical fields (Lvova et al. 2009). They have also been applied in food industry to solve qualitative problems (Bagnasco et al. 2014; Cetó et al. 2013b). A wide variety of recognition elements, combinations thereof and sensor architectures are applied in ET systems and many examples are given in literature: for instance *"in bulk"* biosensors, where the enzymes or other biomolecules are inside the bulk of the electrode, *"inorganic"* sensors, as classic bare electrodes like glassy carbon, graphite or gold etc. and electrodes modified with metal nanoparticles, ion selective sensors (ISEs) covered with a PVC membrane or electrodes with catalysts (Cetó et al. 2012; Gutierrez et al. 2008; Wilson et al. 2015). In the range of surface modified electrodes the possibilities are almost infinite, from nanomaterials to biomolecules, e.g., carbon nanotubes, nanoparticles, enzymes, aptamers and a combination of these modifiers citing just a few (Cipri and del Valle 2014; Ocaña et al. 2014; Pacios et al. 2009). Most of such materials have been exploitable for ETs systems. Due to the nature of the ET there is no need for high selectivity. The key point is a good stability of the sensor response at least through a set of samples but also ideally through days. Also enzymes have been used as a detection element in ETs. Glucose oxidase was used for glucose determination in the presence of common interferents of the enzyme (A.Gutés

2006). Urease (and creatinine deiminase) was used for the determination of urea (or urea plus creatinine) in kidney related samples (Gutes et al. 2005; Gutierrez et al. 2007). Tyrosinase, peroxidases (Sapelnikova et al. 2003; Solna et al. 2005; Tønning et al. 2005) and also laccase were applied for resolution of phenol mixtures (Cetó et al. 2013a; Cetó et al. 2012). Acetylcholinesterases (Tønning et al. 2005) and cholinesterase (Sapelnikova et al. 2003; Solna et al. 2005) were applied to discriminate between different pesticides in their inhibition reaction (Valdes-Ramirez et al. 2009). Such ET systems using biomolecules as enzymes for the detection have received the name Bioelectronic Tongues (BioETs) (Tønning et al. 2005) and were most recently reviewed by Peris and Escuder-Gilabert (Peris and Escuder-Gilabert 2013) and Ha and coworkers (Ha et al. 2015). It is known that enzymes are advantageous due to their inherent, higher specificity and the reduction of the activation energy necessary to drive a desired chemical reaction compared with non-enzymatically catalysed reactions (Berg et al. 2002). Both factors decrease (but do not abolish) the risk of interfering analytes being detected. Recently, there has been a lot of interest in redox enzymes and their applications for electrochemical biosensors, biofuel cells and bioelectrosynthesis (Katz and Willner 2004; Meredith and Minteer 2012; Osman et al. 2011; Rabaey and Rozendal 2010). One focus lies on the establishment of a direct electronic communication between electrodes and enzymes called direct electron transfer (DET) enabling biosensors or biofuel cell electrodes to operate at low or no overpotential with respect to the redox potential of the enzyme leading to increased cell voltages when applied to biofuel cells and when applied to biosensors, to mediator-less, third generation biosensors with decreased problems of interfering species being non-enzymatically detected. Furthermore often toxic and diffusive redox mediators shuttling electrons between enzymes and electrodes can be avoided when working with DET (Leech et al. 2012; Wang 2008). One of the enzymes for which a lot of interest has been shown in the field of biosensors and biofuel cells is cellobiose dehydrogenase (CDH) as it has been shown DET for a variety of substrates including analytically relevant sugars as glucose and lactose (Ludwig et al. 2013a).

CDH is an extracellular oxidoreductase secreted by wood degrading fungi. It is involved in the degradation process of cellulose from wood. The natural substrate is cellobiose, which is

a decomposition product from cellulose. (Henriksson et al. 2000; Ludwig et al. 2010; Zamocky et al. 2006) CDH oxidises cellobiose and reduces lytic polysaccharide monooxygenases (LPMOs), which in its reduced form supports the decomposition of cellulose as was recently found out (Beeson et al. 2011; Eibinger et al. 2014; Langston et al. 2011; Phillips et al. 2011).

CDH consists of two separate domains connected by a flexible polypeptide linker. These two domains consist of a larger, flavin adenine dinucleotide (FAD) containing flavodehydrogenase domain (DH_{CDH}) (Hallberg et al. 2002) and a smaller heme *b* containing cytochrome domain (CYT_{CDH}) (Hallberg et al. 2000). DH_{CDH} is catalytically active and responsible for the oxidation of the substrate leading to a fully reduced FAD cofactor located in the DH_{CDH} (Jones and Wilson 1988). The electrons can be transferred by an internal electron transfer pathway (IET) to the CYT_{CDH} reducing the heme *b* cofactor (Igarashi et al. 2002). The CYT_{CDH} acts as an electron transfer protein between DH_{CDH} and the natural electron acceptor (LPMOs, see above) or an electrode surface (Ludwig et al. 2013a). CDHs are expressed by fungi from the dikaryotic phyla of Basidiomycota and Ascomycota and were phylogenetically classified into class I and class II respectively (Zamocky et al. 2006). Depending on the origin the biochemical properties, as size (usually between 80-100 kDA), isoelectric point (usually below pH 5) substrate spectrum and pH optimum, of CDHs can vary (Ludwig et al. 2010). All CDHs prefer cellodextrines and cellobiose as substrates, but also convert lactose (Zamocky et al. 2006). Some class II CDHs also show activity for glucose (Harreither et al. 2011; Henriksson et al. 1998; Zamocky et al. 2006). Next to differences in substrate specificities recently we found out that the activity of especially class II CDHs also depends on the presence of cations. Especially divalent cations as Ca^{2+} at millimolar concentrations were found to enhance the activity of CDH possibly by enhancing the rate limiting IET by screening negative charges being present at the interfaces of both domains decreasing the distance between the two domains. The activity of *Mt*CDH (class II) was found to be tunable most by Ca^{2+} with increases of around 5 times of its original activity at its optimal pH 5.5 when adding 50 mM Ca^{2+} (Kielb et al. 2015; Kracher et al. 2015; Larsson et al. 2000; Schulz et al. 2012).

The origin dependent different preferences for the substrate (lactose vs. glucose) and the varying dependence of the activity of CDH on cations make CDH a good candidate to build a sensor array to be exploited for a BioET system. CDH has already been applied in a BioET like setup, however in a rather selective manner using other enzymes as cholinesterase, tyrosinase and peroxidase to detect pesticides and phenols with low extend of cross-responses between the electrodes (Solna et al. 2005). However, the use of additional, unmodified electrodes and the rather high selectivity make both terms, Bio and ET questionable in this context. This work aims to show the feasibility of BioET using CDHs from different fungi as recognition elements. To make this possible we took advantage of the power of data analysis in ETs systems and the sensing and DET properties of CDH. To show how the system works two sugars (lactose and glucose) and one activity modulating cation (Ca^{2+}) were chosen as targets. A system like this can be potentially interesting for applications in the food and dairy industry, detecting levels of lactose in e. g. milk or lactose free milk. The Ca^{2+} and glucose content of milk might be of interest to detect possible adulteration of milk (Walstra et al. 2014). In previous studies third-generation biosensors based on CDH to detect lactose in milk and in a dairy processing plant were shown to reliably measure levels of lactose with only dilution necessary as a sample preparation step (Glithero et al. 2013; Safina et al. 2010; Stoica et al. 2006; Yakovleva et al. 2012). Another advantage of this ET system is the non pre-treatment of the samples, giving an advantage for the producers to have a real time or in production line analysis.

2. Experimental

2.1 Reagents and Instruments

Sodium chloride (NaCl), 3-(N-morpholino)propanesulfonic acid (MOPS), lactose, D-glucose, ethanol (EtOH), sulphuric acid (H_2SO_4), hydrogen peroxide (H_2O_2) and 6-mercapto-1-hexanol 97% were purchased from Sigma Aldrich (St. Louis, MO, USA), calcium chloride ($CaCl_2$) was

purchased from Merck KgaA (Darmstadt, Germany), dialysis membranes (Spectrapor, MWCO 12-14 kDA) were purchased from Spectrum Medical Industries (CA, USA). CDH from *Myriococcum thermophilum* (*Mt*CDH) (Zamocky et al. 2008) and a CDH variant from *Corynascus thermophilus* with enhanced activity for glucose (*Ct*CDH C291Y) (Harreither et al. 2012; Ludwig et al. 2013b) were recombinantly expressed in *Pichia pastoris*. CDH from *Neurospora crassa* (*Nc*CDH) was harvested and purified from the fungal culture (Harreither et al. 2011). The CDH preparations were used directly without further dilution and had concentrations of 7 mg/ml for *Mt*CDH, 18.8 mg/ml for *Ct*CDHC291Y and 8.4 mg/ml for *Nc*CDH. The concentrations of the enzymes were determined photometrically converting the absorption measured at 280 nm to a protein concentration by using the calculated absorption coefficients based on the amino acid sequences. The buffer used to perform the experiments was a 50 mM MOPS pH 6.7 adjusted to an ionic strength of 63 mM with NaCl. A pH of 6.7 and an ionic strength of 63 mM were chosen to potentially mimic the conditions present in cow's milk (Walstra et al. 2014).

The voltammetric analyses were performed with an EmStat2 PalmSens potentiostat using three modified gold electrodes as working electrodes (WE), a saturated calomel electrode (SCE) as a reference electrode and a platinum flag as an auxiliary electrode.

2.2 Preparation of the different CDH-biosensors

Polycrystalline gold electrodes (diameter=1.6 mm, BASi, West Lafayette, IN, USA) were cleaned by incubation in Piranha solution for 2 min (1:3 mixture of conc. H_2O_2 with H_2SO_4. (**Careful, the compounds react violently and highly exothermic with each other**), polished on polishing cloths with deagglomerated alumina slurry of a diameter of 1 µm (Struers, Ballerup, Danmark), sonicated in ultrapure water for 5 min and electrochemically cleaned in 0.5 M H_2SO_4 by cycling 30 times between 0.1 V and 1.7 V vs. SCE at a scan rate of 300 mV/s. The sensors were modified with a self-assembled monolayer (SAM) by immediately after rinsing with water, immersing the electrodes in a 10 mM ethanolic solution of 6-mercapto-1-hexanol

overnight at room temperature. The electrodes were gently rinsed with ultrapure water and excess liquid was shaken off. On each of the three electrodes forming the ET 5 µL of a CDH solution, either *Mt*CDH, *Nc*CDH or *Ct*CDHC291Y was dropped on the SAM modified gold electrode surface and entrapped by covering it with a pre-soaked dialysis membrane, which was fixed with a rubber O-ring and Parafilm (Bemis, Neenah, WI, USA) as described by Haladjian and coworkers (Haladjian et al. 1994) and as shown in Fig. S1 (Supplementary material).

2.3 Measurement procedure

The voltammetric cell contained the three working electrodes (WEs) forming the sensor array, a reference and an auxiliary electrode. Each WE of the sensor array was measured independently and successively after each other connecting them to the single channel potentiostat. Stock solutions of lactose (5 mM), glucose (5 mM) and $CaCl_2$ (10 mM) were diluted with 50 mM MOPS/NaCl buffer, pH 6.7, to obtain solutions with varying concentrations of lactose, glucose and $CaCl_2$. For the characterisation and identification of the linear ranges of each of the biosensors used in the array calibration curves with concentrations between 0 and 7 mM for lactose, 0 and 7 mM for glucose and 0 and 50 mM for $CaCl_2$ were used. The concentrations for training and testing the ANN ranged from 0 to 250 µM for lactose and glucose and from 0 to 10 mM for Ca^{2+}. 27 samples were distributed in a simple 3-level factorial design[1] for training and 10 samples were randomly distributed along the experimental domain[2] for external test (Fig. 1).

The cyclic voltammetric measurements were performed under nitrogen atmosphere at room temperature with samples being degased for 10 min with nitrogen prior to the measurement. The working potential was swept between -0.3 V and 0.15 V *vs.* SCE at a scan rate of 20 mV/s and a step potential of 2 mV.

[1] A 3-level factorial design is a design with points of interest organized in a cube 3x3x3.

[2] The experimental domain is the range of concentrations used to train the ANN and the test has to stay inside the domain otherwise they would be insignificant.

2.4 Building the ANN model

The data analysis of the measurements was carried out using a multivariate calibration process. This process was based on an Artificial Neural Network (ANN) as response model. As explained in Section 2.2 a batch of 37 samples was prepared and divided in two groups (27+10), this division was made to train the model using the group of 27 and to test it with the group of 10. The test samples are useful to determine the prediction ability of the ANN. The samples were distributed randomly during the measurements to avoid any history effect.

The architecture definition of the ANN was configured and optimised based on our group's previous experience with ETs formed by amperometric sensors (Fig. 2). The optimisation included the number of neurons in the hidden layer in a range between 4 and 12, the number of output neurons was fixed to 3 (the number of target molecules) and 4 transfer functions (*logsig*: log-sigmoidal, *tansig*: hyperbolic tangent sigmoid, *purelin*: linear and *satlins*: saturated-linear) were assayed for each layer (input and output). Another optimisation step was provided from the pre-processing of the voltammetric data. The voltammograms were used in their full size unfolding them and joining the signals from every sensor to a "single-sensor-like" signal per every sample. Since this has generated tens of thousands of data points a pre-processing step consisting of wavelet compression was used. The pre-processing/compression step allowed the decrease of data to be managed from the software from 48 600 to 186 values, with a Daubechies wavelet function (*db4*) and a compression level of 3.

To evaluate the goodness of fit of the ANN model, the smallest MSE (mean squared error) from the test sample set was taken. The prediction abilities, instead, were evaluated from the linear regression of the comparison graphs of obtained (y) vs. expected (x) concentrations with desired slope of 1 and correlation coefficient close to 1 for the three target molecules.

2.5 Software

The voltammetric data were acquired by using PSTrace 4.4 (PalmSens, Utrecht, The Netherlands) software. Neural Network processing was developed by the authors by MATLAB 7.0 (Mathworks, Natick, MA, USA), using its Neural Network Toolbox (v. 3.0). The graphs were made with Sigma Plot 12 (Systat Software Inc., California, USA).

3. Results and discussion

3.1 Characterisation of each CDH biosensor

The sensor array used for the ET consisted of three different CDH modified gold/SAM electrodes – one was modified with *Mt*CDH, one with *Nc*CDH, and one with *Ct*CDHC291Y, expecting different substrate specificities depending on the enzyme origin. Before developing the ET application, the integrity and linear ranges for each of the biosensors versus each of the three analytes of interest, lactose, glucose and Ca^{2+} were determined. In Fig. 3 the cyclic voltammograms of the gold/SAM electrodes modified with either *Mt*CDH, *Nc*CDH or *Ct*CDHC291Y are shown. In the absence of substrate, clear redox waves originating from the oxidation and reduction of the heme *b* cofactor located in the CYT_{CDH} is visible. The midpoint potentials range between -153 mV vs. SCE for *Mt*CDH, -144 mV vs. SCE for *Nc*CDH and -148 mV vs. SCE for *Ct*CDHC291Y, which are close to literature values (Coman et al. 2007; Harreither et al. 2012; Sygmund et al. 2012). The additional oxidative redox wave present for *Nc*CDH at -210 mV vs. SCE might originate from the oxidation of the FAD cofactor located in the DH_{CDH} as found out to be possible recently (Schulz et al., in manuscript). The peak separations between the anodic and cathodic peak potentials vary between 42 mV and 52 mV and thus lay between a solution and a surface confined redox process typical for thin layer protein electrochemistry (Haladjian et al. 1994; Laviron 1979). When lactose as the standard

substrate is added clear catalytic waves can be seen for all CDH modified electrodes proving they are catalytically active. For *Mt*CDH and *Ct*CDHC291Y, the catalytic waves start at potentials around the oxidation peak potential the CYT_{CDH} peak indicating DET from the CYT_{CDH} domain. For *Nc*CDH it seems that there are traces of catalysis at potentials already below the oxidation potential of the CYT_{CDH} peak indicating a potential DET from the DH_{CDH} as found out to be possible recently (Schulz et al., in manuscript). To determine the linear measuring ranges, the response of each CDH biosensor to varying analyte concentrations of lactose, glucose and Ca^{2+} was determined, as shown in Fig. 4. The investigation with Ca^{2+} as analyte was performed in the presence of 7 mM lactose, since Ca^{2+} is not a substrate for CDH but only potentially increases the existing catalytic currents by affecting the interdomain electron transfer. As shown in Fig. 4 all CDH biosensors tested respond to lactose. The best responding biosensors are the ones modified with *Nc*CDH and *Ct*CDHC291Y, possibly because their pH optima for DET of 5.5 (Kovacs et al. 2012) and 7.5 (Harreither et al. 2012) respectively are close to the investigated pH of 6.7. The pH optimum for DET of *Mt*CDH is also at pH 5.5 (Harreither et al. 2007) but its decline of activity with increasing pH is steeper compared to that of *Nc*CDH (Harreither et al. 2007; Kovacs et al. 2012) possibly explaining the comparable low response of the *Mt*CDH biosensor to lactose. The upper linear measuring range for lactose can be estimated to reach 500 µM.

When looking into glucose as substrate (Fig. 4, middle row) clearly the *Ct*CDHC291Y variant designed for high activity with glucose responds best to glucose with a linear measuring range to up to 260 µM. When looking into Ca^{2+} as analyte the *Ct*CDHC291Y and *Mt*CDH modified biosensors are sensitive to additional Ca^{2+}. The absolute currents are higher for *Ct*CDHC291Y, since its activity in the absence of Ca^{2+} is already higher than that for *Mt*CDH. However, looking into the relative increases of the catalytic currents in the presence of 50 mM Ca^{2+} increases of around 6.3 times for *Ct*CDHC291Y and 4.5 times for *Mt*CDH show similar dependencies for both enzymes on additional concentrations of Ca^{2+}. The dependency of the activity of *Mt*CDH on additional [Ca^{2+}] is comparable to what has been found in other studies done at pH 5.5 and 7.5 (Kracher et al. 2015; Schulz et al. 2012). The activity of the glucose

variant, *Ct*CDHC291Y, has not been studied before in the presence of Ca^{2+} but when comparing its activity with that of the wild-type *Ct*CDH in solution with cytochrome *c* as electron acceptor, the Ca^{2+} induced activity increase found here is around twice as high (Kracher et al. 2015). The nearly independence of the activity of *Nc*CDH on additional [Ca^{2+}] compares well with the literature, where no increase for *Nc*CDH was found when investigated in solution with cytochrome *c* as electron acceptor (Kracher et al. 2015). The linear ranges found here for the detection of Ca^{2+} range up to around 10 mM.

In summary, each investigated biosensor responds differently to the investigated analytes, which is a desired departure point for any ET design. This cross-response pattern was used to create a BioET with the help of an artificial neural network to resolve mixtures of all three analytes containing varying concentrations of lactose and glucose between 0 and 250 µM and between 0 and 10 mM for Ca^{2+}. The BioET contained the three biosensors modified with *Mt*CDH, *Nc*CDH, or *Ct*CDHC291Y and an auxiliary and a reference electrode. The response of the ET to a set of 27 training samples and 10 test samples containing all three analytes was determined an analysed as input data for building the response model.

3.2 ANN response model

The training method used in the Artificial Neural Network was described in Section 2.4. To define the best architecture 144 different configurations were evaluated (product of the number of neurons in the hidden layer, the tested transfer functions in the hidden layer and the transfer functions in the output layer). The best result was as follows: 9 neurons in the hidden layer with the transform function *"logsig"* and 3 neurons in the output layer with a *"purelin"* as transform function. With this configuration the responses of the 10 test samples with known concentrations of lactose, glucose, and Ca^{2+} were then used to compare their calculated concentrations according to the ANN with their real concentrations. The correlations between predicted (by the ANN) and expected concentrations for all analytes are shown in Fig. 5, in this case for the external test set, the samples not intervening for the training process. Considering

that the samples are mixtures of similar and/or interfering compounds and the typology of the sensors used satisfactory R^2 values were calculated especially for lactose (0.975) and Ca^{2+} (0.945). For glucose a rather low R^2 of 0.726 was obtained. Despite the R^2 value for glucose being rather low, the presence of the data related to glucose in the model supports the prediction of the other two targets, lactose and Ca^{2+}. This behaviour could be explained considering that in the array there is only one biosensor to detect glucose, which can lead to a little less accurate detection; moreover for the *Ct*CDH291Y modified electrodes, the enzyme is involved also in the conversion of lactose resulting in that the active site of the enzyme is partly occupied by the preferred substrate, giving a little lower sensitivity for glucose. To demonstrate how keeping the data related to glucose supported the prediction of lactose and Ca^{2+}, a few tests were run using 2 neurons for the output layer (data not shown), to predict only lactose and Ca^{2+} while keeping the rest of the configuration as it was for the original ANN. This resulted in significantly lower regression values showing that the ANN was less able to make a good prediction for lactose and Ca^{2+} when excluding the glucose related data. Also the prediction was completely random, being different every time the software was run. This behaviour of the ANN led us to the conclusion that even if the glucose concentration predictions were poor, those data were essential for the complete prediction of the three analytes. As an example of the goodness of the prediction model the samples, used as external test to verify the ANN, are grouped in Table 1 showing the expected and predicted concentrations. The deviations between predicted and expected concentrations are on average +6.9% for lactose, +4.8% for Ca^{2+} and +12.3% for glucose, a highly reliable result, derived in this case from the different specificities shown by the different CDH enzymes used.

4. Conclusions

In this work we show the feasibility of a novel BioET system integrating bioinformatics for data treatment and bioelectrochemistry for smart analyte detection. In the present system we utilize

the cross response of CDHs from different origins to the substrates lactose and glucose and the interfering compound Ca^{2+} to resolve mixtures of these analytes. The system operates in a DET mode allowing analyte detection at reduced potentials. After data treatment with an artificial neural network the BioET was able to successfully predict the concentrations especially for lactose and Ca^{2+} in artificial samples with an R^2 value of 0.975 and 0.945 respectively and a low deviation from the expected concentration values of +6.9% for lactose and +4.8% for Ca^{2+}. A BioET system like this, using the DET capabilities of CDH from different origins as sensor modifiers is a complete novelty. The great variety of CDHs may allow the system to be tailored to detect also other analytically relevant sugars as e.g. maltose or cellobiose. The findings are of potential interest for new biosensor applications for the food and dairy industry.

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Table 1. Comparison of expected and predicted concentrations of the external test samples for the three analytes.

Lactose (µM)		**Glucose (µM)**		**Ca^{2+} (mM)**	
Expected	*Predicted*	*Expected*	*Predicted*	*Expected*	*Predicted*
2.03E+02	2.25E+02	9.69E+01	6.57E+01	3.83E+03	4.85E+03
5.81E+01	7.40E+01	1.12E+02	1.81E+02	1.89E+03	1.53E+03
2.14E+02	2.25E+02	2.43E+02	2.53E+02	7.24E+03	8.11E+03
1.13E+02	1.05E+02	5.97E+01	4.01E+01	7.93E+03	8.98E+03
5.86E+01	6.49E+01	8.75E+01	1.43E+02	4.14E+03	5.73E+03
4.14E+01	6.22E+01	9.82E+01	1.54E+02	2.67E+03	3.72E+03
4.00E+01	7.00E+01	1.95E+02	2.90E+02	4.70E+02	1.18E+03
1.87E+02	2.22E+02	6.47E+01	2.35E+01	9.42E+03	9.68E+03
2.19E+02	2.33E+02	1.88E+02	1.76E+02	6.01E+03	5.41E+03

Captions for Figures

Fig. 1. Distribution of the training (blue) and test (red) concentrations of lactose, glucose, and Ca^{2+} used to train and test the Artificial Neural Network (ANN).

Fig. 2. Scheme of the ANN architecture used. The numbers surrounded by the red circles are the numbers of neurons used in each layer.

Fig. 3. Cyclic voltammetric characterisation of the enzyme modified electrodes used for the construction of the electronic tongue. CDH was entrapped under a dialysis membrane on mercaptohexanol modified gold electrodes. From top to bottom it is possible to see the behaviour of *Mt*CDH (A), *Ct*CDHC291Y (B) and *Nc*CDH (C) in a 50 mM MOPS buffer solution at pH 6.7 in the absence (solid line) and in the presence of 250 μM lactose (dashed line). All experiments were performed at a scan rate of 20 mV/s with a SCE reference electrode and a Pt flag as counter electrode.

Fig. 4. Calibration graphs of the *Mt*CDH, *Ct*CDHC291Y and *Nc*CDH based biosensors obtained for the three analytes lactose (top, A, B), glucose (middle, C, D) and Ca^{2+} (bottom, E, F). On the left, the fully investigated concentration ranges are shown; on the right, only the linear concentration ranges are shown.

Fig. 5. Comparison graphics of the "expected vs. predicted" concentrations for the target analytes; lactose (A), glucose (B), and Ca^{2+} (C) calculated by the ANN using the external test set samples with known, expected concentrations.

Figure 1

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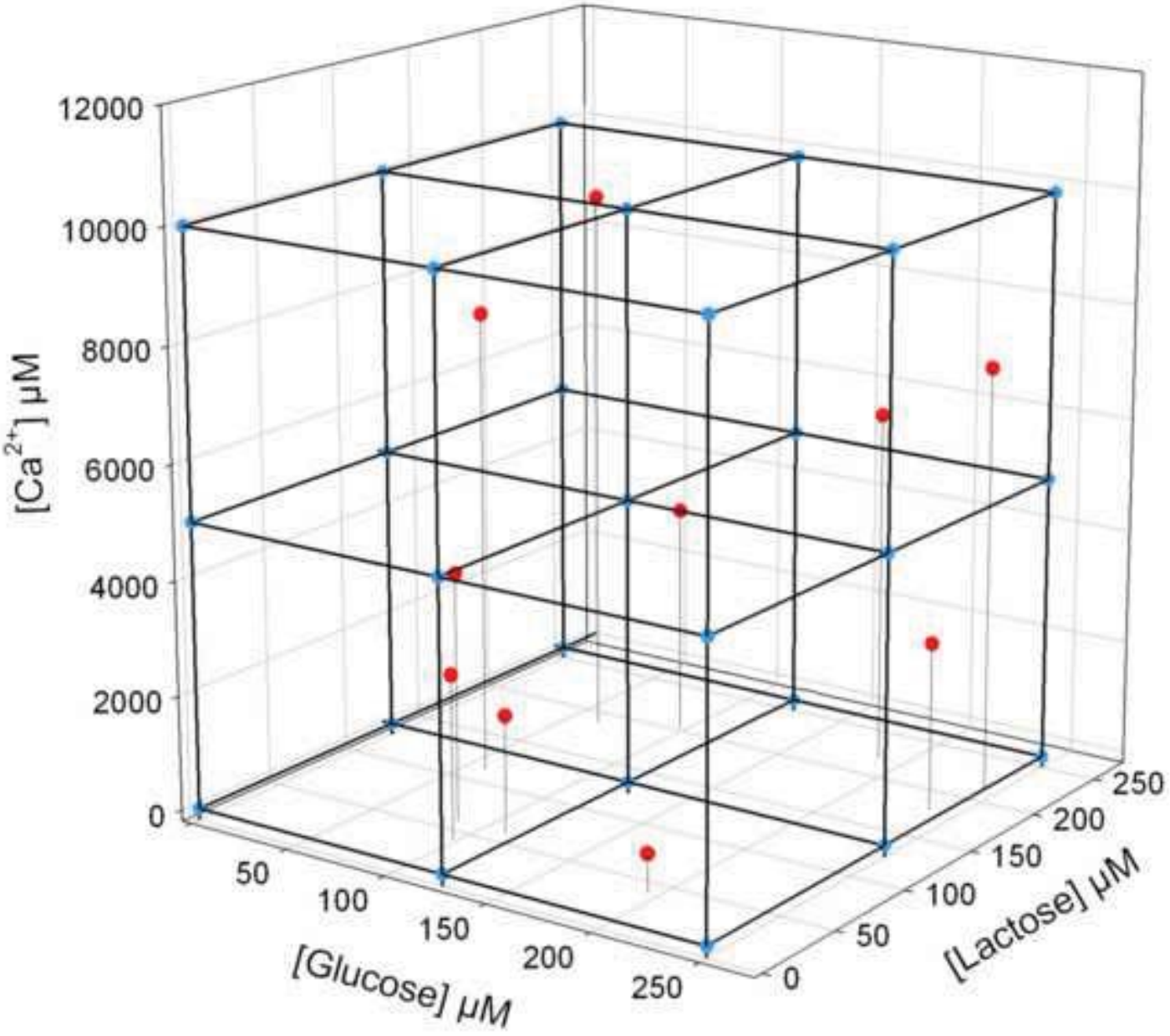

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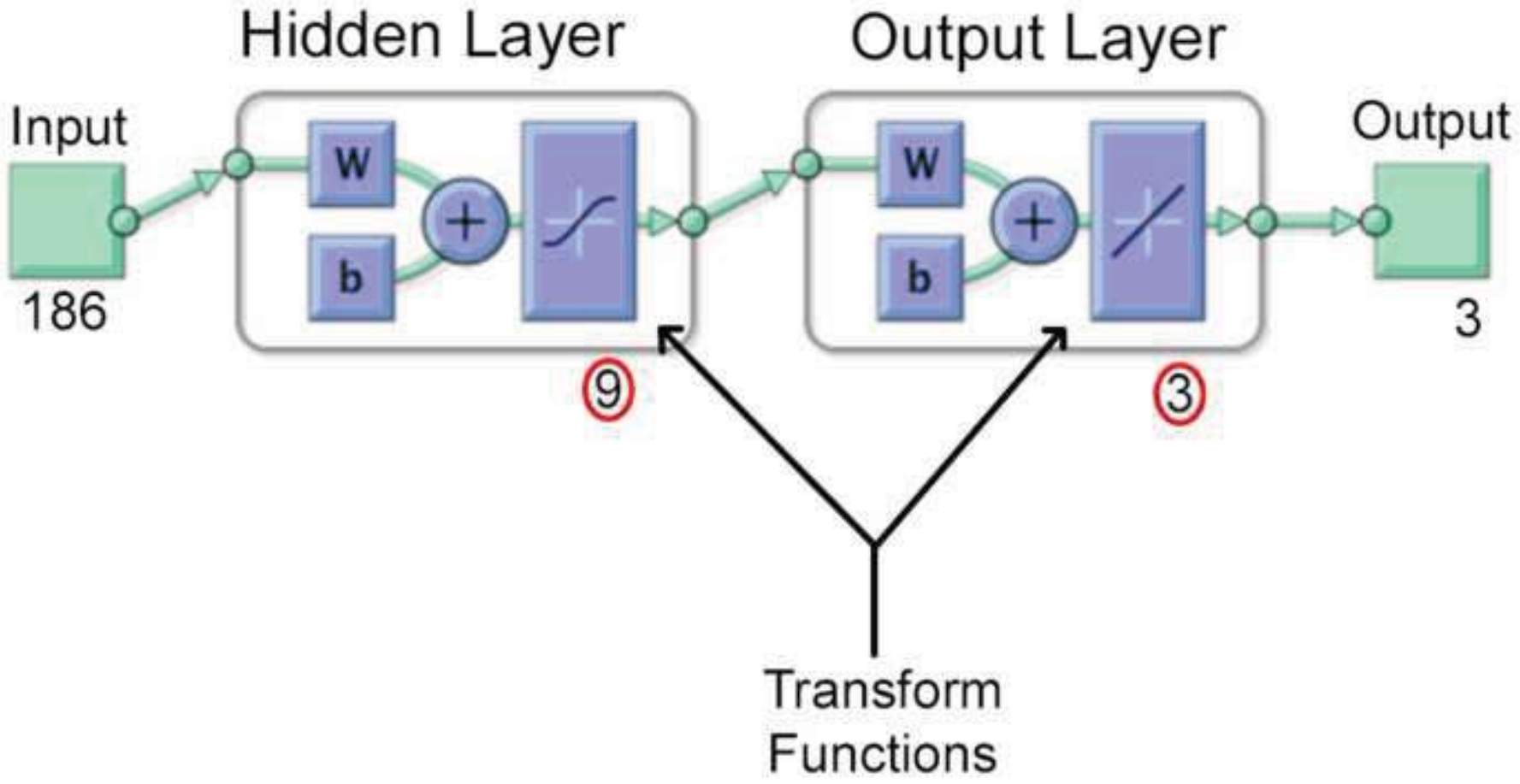

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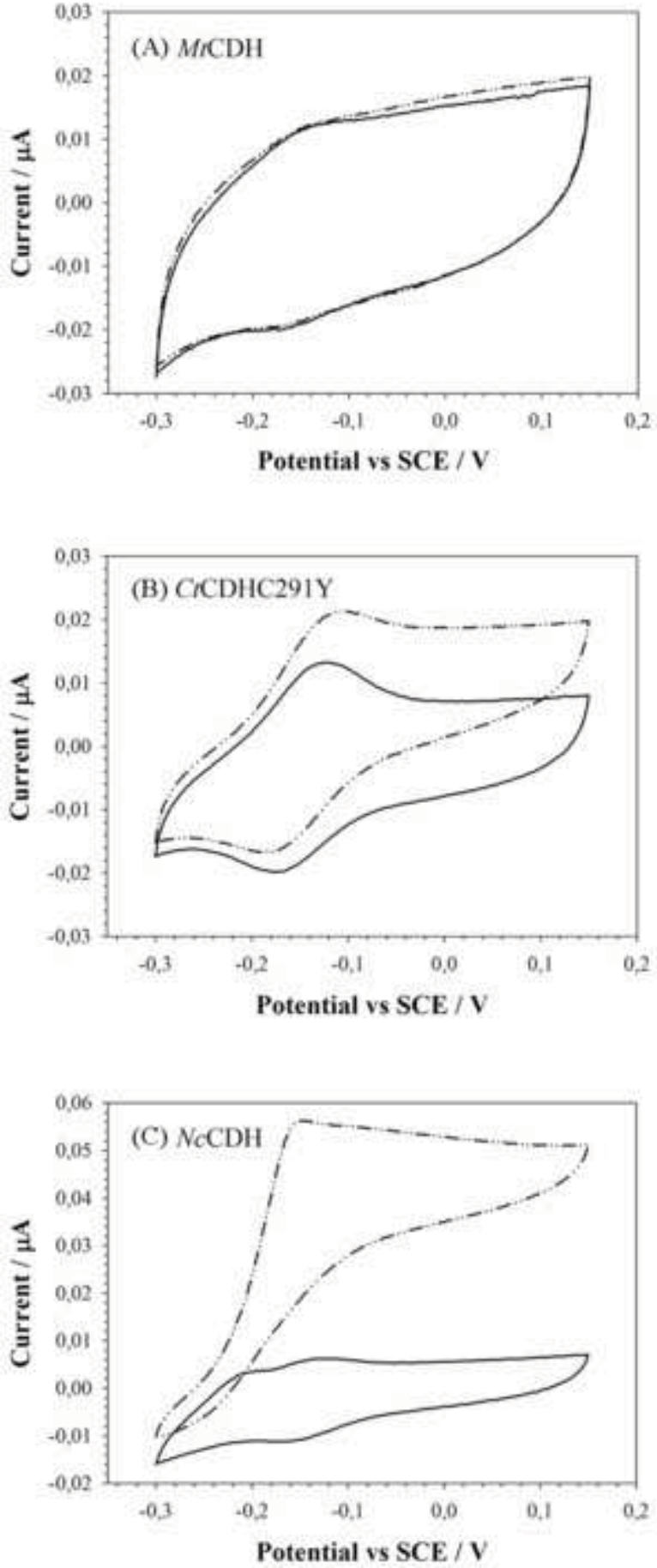

Figure 4

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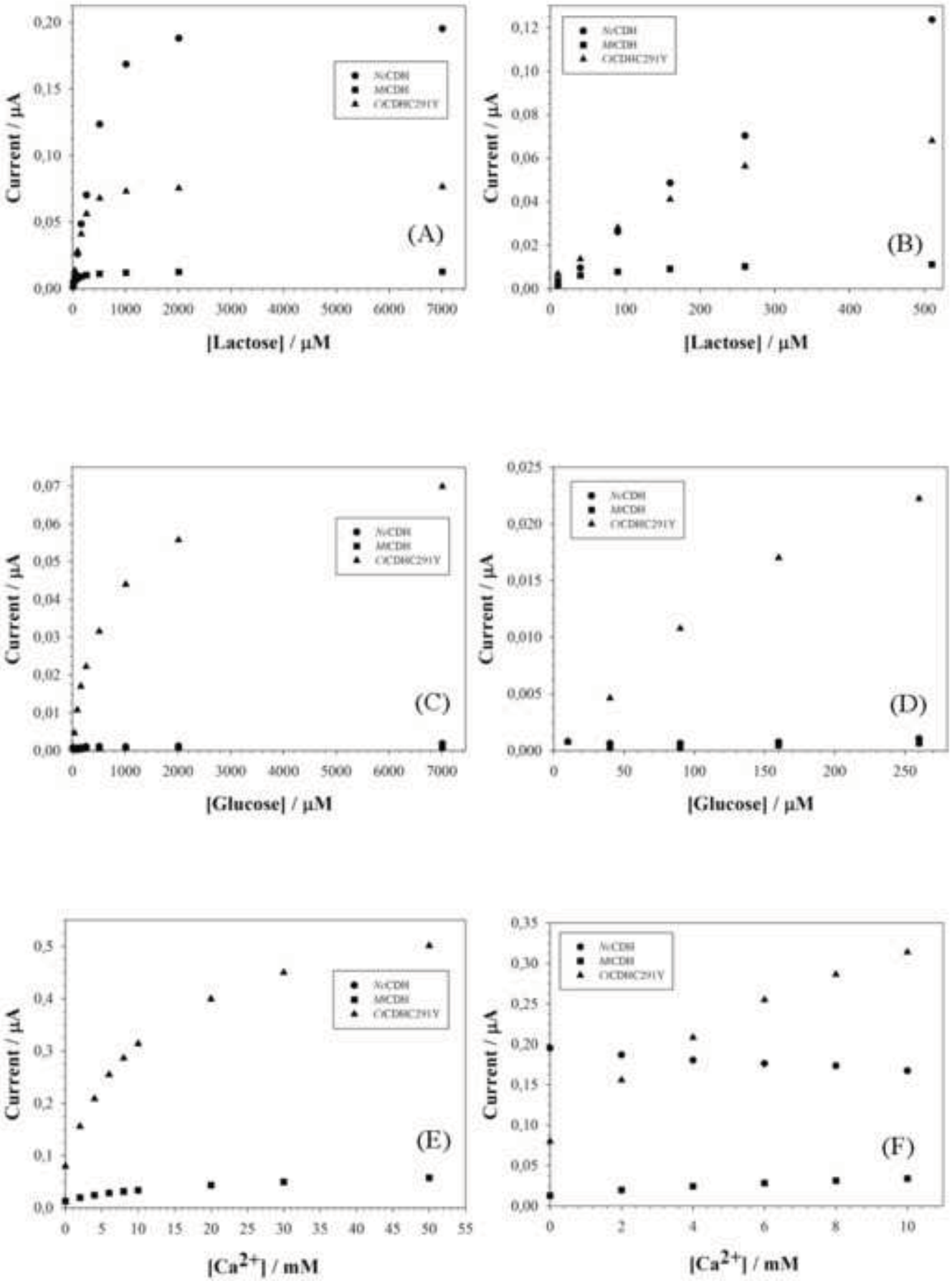

Figure 5
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