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NOVEL POTENTIOMETRIC BIOSENSOR FOR AMINO ACIDS DETECTION

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Novel potentiometric biosensor for amino acids detection

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Abstract. The development of a novel paper-based enzymatic sensor for potentiometric detection of amino acids is presented. The sensor consists of a platinized paper that acts as a redox-sensitive substrate and a layer of Nafion on top of which the enzyme L-Amino Acid Oxidase (LAAO) is immobilized into Chitosan polymeric matrix. Nafion acts as perm-selective barrier, avoiding the transport of negatively charged species while being permeable to neutral species such as H_2O_2. The composition of the biosensor was optimized and the response to different amino acids with different side chains was explored. Nonpolar amino acids with aromatic groups (Phe, Tyr and Trp) reported the highest sensitivity (-32.3, -56.1 and -45.3 mV/decade respectively) within the 10^{-4} to 10^{-3} M linear range, i.e., the normal clinical range of amino acids present in human plasma. All other screened amino acids display almost negligible response. The only exception is Cys, which shows response due to its own redox characteristics. Thus, since the concentration of Cys may be independently determined, the herein developed sensor could find possible application for the detection of total aromatic amino acid content.

1. INTRODUCTION

Amino acids play a central role in biological systems both as building blocks of proteins and as intermediates in metabolic processes. Twenty-two L-enantiomers of amino acids are building all existing proteins of the human body (proteinogenic amino acids) and the way they are linked by peptide bonds inside a protein determines the function of a polypeptide chain. In addition, the amino acid sequence contains important information, since it determines the protein stability and the several structural orders (e.g., secondary, tertiary, etc.) that will result in the spatial arrangements that give way to the protein biochemical reactivity. A group of these compounds, usually called essential amino acids, cannot be synthesized by the human body and therefore must be afforded through nutrition. In any case, whether it is due to nutritional deficiencies or metabolic disorders (e.g., increased concentration of some amino acids is associated with lack on enzymes included in their metabolic pathway), the presence of abnormal levels (either deficiency or excess) of amino acids in the organism, almost invariably represents a serious health problem [1–4]. For this reason, special attention is devoted to monitoring amino acids' levels in blood. For example, deficiency of enzyme phenylalanine hydroxylase that hydrolyses Phenylalanine (Phe) to Tyrosine (Tyr), leads to the abnormal accumulation of Phe in the body and health disorder called phenylketonuria (PKU) [1,5,6]. Similarly, malfunction of the branched chain α-keto acid dehydrogenase complex leads to the maple syrup urine disease (MSUD), disorder characterized by increased concentrations of L-branched-chain amino acids (L-leucine, L- isoleucine and L-valine) in blood and urine [7,8]. If not treated on time, these diseases cause severe mental disorders in humans. However, if neonatal diagnosis is performed, appropriate treatment that usually requires special diets, can be done.

Beyond the substantial clinical relevance, amino acids find applications in a plethora of different fields, such as food technology and biotechnology (e.g. the production of biopharmaceuticals, etc.), all of which requires qualitative and quantitative measurements of amino acid content. In biochemical structural analysis, for example proteins are hydrolyzed and amino acid composition is used to confirm amino acids' sequence inside the polypeptide chain [9].

In addition, amino acid content is essential in any (bio)technological process involving cell cultures, since

many bacteria and yeasts require some type of amino acid as a source of nitrogen. Cell cultures are used to produce proteins that have application as biopharmaceuticals. For example, the concentration of essential amino acids, which determines maximum cell density that can be achieved, must be carefully optimized and controlled during the cell growth in the culture media. In this case, glutamine is particularly important because it degrades very quickly inside the culture media and therefore must be supplemented [10–12].

Similarly, the assessment of amino acids' concentration is also important in fermentation processes, such as in wine production. Nitrogen presented in grapes in form of amino acids is used by yeast during the alcoholic fermentation process [13]. This nitrogen is called yeast assimilable nitrogen and represents part of nitrogen that can be used by wine yeast during the fermentation [14]. If concentration of nitrogen is not sufficient, yeast growth and fermentation speed will be limited and amino acids have to be supplied to the grape must necessarily [15]. Therefore, measurement of nitrogen concentration, i.e. amino acids' concentration during the fermentation is of huge importance for wineries.

Last but not least, essential amino acids are also used as additives to animal feeds, and control of their total amount enables at the same time the high nutritional value of feeds and more economical production [16,17]. All in all, because of the importance of proteins in any kind of biological process (the implicit relevance of amino acids as building blocks of proteins), the determination of the levels of amino acids in different media is extremely important.

For this reason, there is significant interest on the development of simple and accurate tools for monitoring amino acids levels in biological matrices. Contrasting with this need, current methods of determination of amino acids are usually complex, expensive and time-consuming. In the diagnosis and monitoring of diseases related with metabolic disorders of amino acids, for example, separation techniques such as the high pressure liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS-MS) are often used [5,7,8,18,19].

Electrospray ionization coupled to some form of mass spectrometry (ESI-MS) or gas chromatography with a similar detection scheme can be also used, but in both cases derivatization of the amino acids is required. In essence, available method currently employed in analytical lab suffer from disadvantages such as high cost of analysis and complex sample preparation. For this reason, these techniques are unsuitable for decentralized monitoring of amino acids' levels, which is one of the main trends in modern analytical chemistry. Several commercially available kits for determination of the blood spot profiles are available, but their high price prevents their widespread use. Therefore, cost-reduction by using paper-based potentiometric biosensors could have important advantages.

In this report, development of a novel enzymatic approach using paper-based sensors for the potentiometric detection of amino acids is presented. Sensors are constructed by using platinized paper coated with a layer of Nafion, where L-Amino Acid Oxidize (LAAO) is immobilized following different immobilization approaches. LAAO is a specific catalyst for decomposition of L-amino acids to 2-oxo acid, ammonia and hydrogen peroxide as it is shown in the overall reaction (Figure 1) [20,21]. Ammonia or hydrogen peroxide can be potentiometrically detected, so that concentration of amino acid can be indirectly determined. This work will be focused on the selective detection of hydrogen peroxide using Pt electrode coated with Nafion. As it has been published previously [22,23], Pt electrode senses changes of redox potential of solution that happens when hydrogen peroxide is produced in enzymatic reaction. Briefly, on the surface of Pt working electrode, hydrogen peroxide is electrochemically oxidized to water and oxygen, causing a change of its potential. Nafion coating enables higher sensitivity for H_2O_2 compared to bare Pt electrodes, while effect of redox-active negatively charged interferences is reduced [23]. In this work also, complete optimization

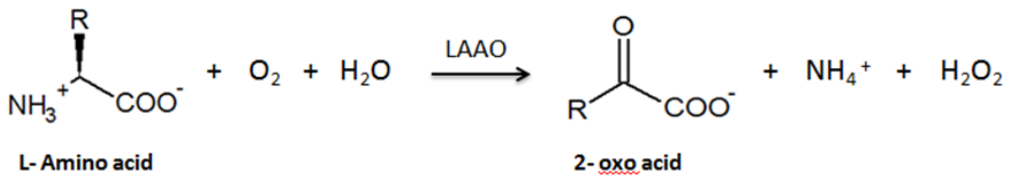

Figure 1. Enzymatic reaction in which L-Amino acid is converted to 2-oxo acid, hydrogen peroxide and ammonium.

of sensor construction and experimental conditions is performed. Application of these sensors for amino acid screening and possibility of their use for decentralized monitoring is discussed.

2. EXPERIMENTAL

2.1 Reagents and Materials

L-Amino Acid Oxidase (LAAO) from Crotalus adamantus (Type I, dried venom, ≥ 0.3 unit/mg), Nafion® 117 solution (5 % wt. in a mixture of lower aliphatic alcohols and water), polyvinyl alcohol (96 % in water), Chitosan (medium molecular weight), polyethylenimine solution (50 % (w/v) in water), polystyren-block-poly(ethylene-ran-butylene)-block-polystyrene (5 wt. % in 1-propanol and dichloroethane), glutaraldehyde solution (25 %, Grade I), hydrogen peroxide (30 wt. % in water) and L-Ascorbic acid were purchased from Sigma-Aldrich.

Amino acids L-Phenylalanine and L-Proline (Bioultra, ≥ 99.0% and ≥ 99.5 % respectively), L-Tryptophan, L-Tyrosine, L-Valine (all reagent grade, ≥ 98%), L-Cysteine (97 % purity) and L-Glutamine (ReagentPlus, ≥ 99 %) were purchased from Sigma-Aldrich. L-Arginine (Bioultra, ≥ 99.5 %) was purchased from Fluka.

Phosphate buffer saline (PBS) was prepared in concentration of 0.1 M and was used in all experiments. All aqueous solution were made using 18.2 MΩ cm^{-1} double deionized water (Milli-Q water systems, Merck Millipore).

2.2 Fabrication of enzymatic amino acid sensor

Whatman® Grade 5 qualitative filter paper was used as a substrate. All electrodes were made by sputtering Pt on one side of filter paper which then was cut into rectangular pieces (20 mm x 5 mm) and placed between two plastic masks. The top mask had a circular window with diameter of 3 mm. A free section of the platinized conductive paper was used as the connection with the potentiometer, while circular window represents a sensing area (Figure 2.1). First, 7 µL of 5 % or 20 % wt Nafion solution was drop-casted on all electrodes and air-dried on room temperature for 3 hours. Thereafter, four different methods for immobilization of L-amino acid oxidase (LAAO) were performed (Figure 2.2).

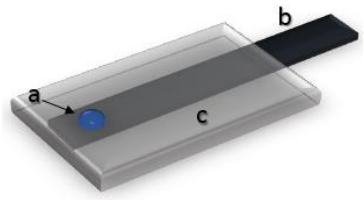

Figure 2.1. Scheme of the potentiometric paper based sensor: a) circular window with drop-casted Nafion membrane; b) free area of platinized paper used as the electric contact; c) plastic mask.

I. Electrodes with a single layer of LAAO

10 µL of solution containing 1 mg/mL of L-Amino Acid Oxidase (LAAO) in distilled water was drop casted on top of the Nafion membrane and the system was left drying in air overnight at 4 °C.

II. Electrodes with amino acid oxidase (LAAO) sandwiched between two Nafion layers

10 µL of solution containing 1 mg/mL of L-Amino Acid Oxidase (LAAO) in PBS was drop casted on top of the Nafion membrane and the system was left drying overnight at 4 °C. However, unlike the previous approach, in this case the layer of enzyme is sandwiched with another layer of Nafion. To do this, 7 µL of 5 % wt Nafion was drop-casted on top of the enzyme layer and the system was left drying in the air overnight at 4 °C.

III. Electrodes with amino acid oxidase (LAAO) embedded into a Chitosan polymeric matrix

1 % wt/V chitosan solution was made by measuring appropriate amount of Chitosan and dissolving it in 1 % wt/V acetic acid. In order to dissolve Chitosan, the solution had to be mildly heated and thoroughly mixed using a vortex. The enzyme cocktail was prepared by dissolving 1 mg of L-Amino Acid Oxidase (LAAO) in 1 mL of 1 % wt polyvinyl alcohol (PVA) solution. This solution was added to the 2 mL of 1 % wt/V Chitosan solution and mixed in vortex until a homogenous solution was obtained. Finally, 8 µL of this solution was drop casted on top of the Nafion membrane and the system was left drying overnight at 4 °C.

IV. Electrodes with amino acid oxidase (LAAO) cross linked with polyethylenimine (PEI) using glutaraldehyde (GTA)

First, 8 µL of 1 % wt polyethylenimine (PEI) solution was drop casted on top of Nafion layer. Thereafter

100 µL of solution containing 1 mg/mL of L-Amino Acid Oxidase (LAAO) in phosphate buffer and 25 µL of 1% wt glutaraldehyde solution were mixed and 8 µL was drop casted on the top of PEI layer. Each drop casting was done after drying underlying film completely overnight at 4 °C.

When not in use, all sensors were stored at 4 °C.

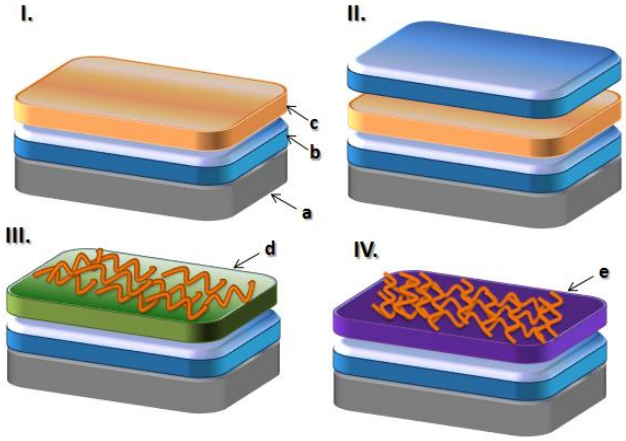

Figure 2.2. Different methods of L-Amino Acid Oxidize (LAAO) immobilization. All sensors are composed of platinized paper (a) covered with a layer of Nafion (b); I. Sensor with a single layer of LAAO (c) on the top of Nafion layer (b); II. Sensor with LAAO sandwiched between two Nafion layers; III. Sensor with LAAO embedded into a Chitosan polymeric matrix (d); IV. Sensor with LAAO cross linked with polyethylenimine (PEI) (e).

2.3 Potentiometric measurements

Potentiometric measurements were performed using standard two-electrode system, containing the fabricated working electrode (sensor) and double junction Ag/AgCl/KCl 3 M (type 6.0726.100, Methrom AG) containing a 1 M LiAcO electrode bridge as reference electrode. All measurements were performed in a 4 mL cell in 0.1 M PBS (pH 7.4) at 25 °C. Electromotive force versus time was recorded using high input impedance EMF16 multichannel data acquisition device (Lawson Laboratories, Inc. Malvern).

3. RESULTS AND DISCUSSION

3.1 In situ detection of amino acids

Preliminary studies were performed in order to assess the type of response with different amino acids by decoupling the enzymatic reaction and the signal detection. For this reason, first experiments were performed by using a Pt electrode coated with a single layer of 5% Nafion as a working electrode, in order to detect *in situ* the hydrogen peroxide generated as a result of enzymatic oxidation of the amino acids. Thus, instead of being immobilized on the electrode, the enzyme was solubilized and used as part of the bulk solution. For that purpose, 0.5 mg/ml solution of L-amino acid oxidase (LAAO) in PBS was prepared and the concentration of any particular amino acid was increased from 10^{-6} M to either 10^{-1} M or 10^{-3} M (depending on the solubility of selected amino acid). Experiments were performed sequentially in separate vessels for each amino acid. Duplicate measurements were performed.

Different types of amino acids belonging to different groups based on their side chain have been tested: a) nonpolar, aromatic chain (Phenylalanine (*Phe*), Tyrosine (*Tyr*), Tryptophan (*Trp*)); b) positively charged chain (Arginine (*Arg*)); c) negatively charged chain (Glutamic acid (*Glu*)); d) –SH group in chain (Cystein (*Cys*)); e) nonpolar, aliphatic chain (Valine (Val)); f) uncharged chain, polar (Proline (*Pro*)). A plot showing the variation of the electromotive force as a function of the logarithm of amino acid concentration (calibration curve) for each of the tested amino acid is shown in Figure 3.

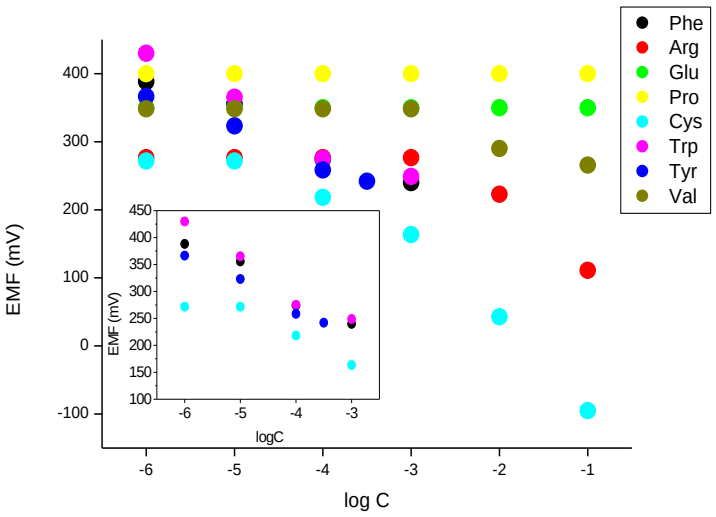

Figure 3. Electromotive force as a function of the logarithm of amino acids' concentration. Inset: Calibration plots for Phe, Trp, Tyr and Cys in the range of concentrations from 10^{-6} M to 10^{-3} M.

This preliminary evidence shows that all aromatic amino acids (group a, Phe, Trp, Tyr) show response in a range of concentrations from 10^{-6} M to 10^{-3} M, with a linear range from 10^{-5} M to 10^{-4} M and sensitivity

values of -80, -90 and -65 mV/decade, respectively. Detection of other groups of amino acids such as Arginine and Valine, was observed only at higher concentrations, i.e. in a range from 10^{-3} M to 10^{-1} M. Glutamic acid and Proline did not yield any signal, while Cysteine showed the broadest linear range from 10^{-5} M to 10^{-3} M with a sensitivity value of -55 mV/decade. However, this distinct potentiometric response of Cysteine originates not only from the detection of hydrogen peroxide, but also from the additional redox process such as oxidation of Cysteine to Cystine on the Pt surface. Indeed, further blank experiments using the same detection system but without the enzyme showed that, unlike all the other amino acids, the direct addition of Cys produces a redox response. Potentiometric time trace showing the response to the additions of Cys to PBS solution is displayed in Figure 4. An additional control experiment was performed by adding Phe to the same electrodes. No response was detected thus confirming that Phe detection is based only on H_2O_2 production from the enzymatic reaction (Figure 4 Inset). This behaviour of Cys is not surprising, considering that due to the reducing nature of the SH- residues this amino acid is a redox-active molecule.

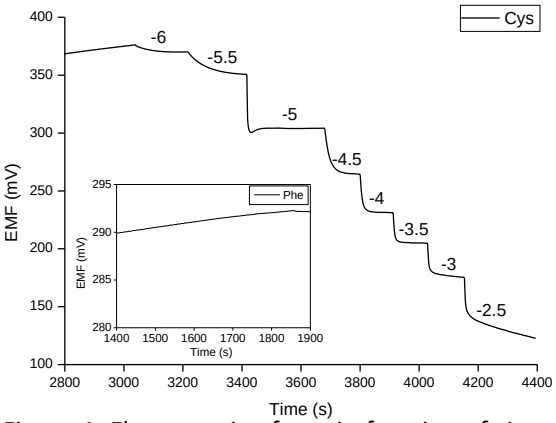

Figure 4. Electromotive force in function of time (time trace) of Cys additions to Pt electrodes covered with Nafion in PBS 0.1 M pH 7.4. Inset: Additions of Phe to the same electrode in the same conditions.

3.2 Selection of immobilization procedure

In order to evaluate the optimum immobilization procedure for the LAAO, additions of Phenyalanine (Phe) to four different sensors were performed. Time trace and corresponding calibration curves obtained with enzymatic sensors with immobilized LAAO, are shown in Figure 5. From time trace and calibration curves, it can be concluded that the sensor containing LAAO immobilized between two Nafion layers shows the lowest response. This almost negligible response could be ascribed to electrostatic repulsions between negatively charged sulfonate groups of the upper Nafion layer and amino acid presented as zwitter ions. To test this hypothesis, experiments using Arginine (Arg) instead of Phe were performed, since Arg has an additional positive charge in –R chain. In this case, improved, though still weak, response was obtained, probably due to the reduced electrostatic repulsion between negative sulfonate groups of Nafion and Arg, which shows two positive and one negative charge. Sensors with a single layer of Nafion and LAAO showed better but still low sensitivity towards Phe. In this case, reduces response could be ascribed to the leaching of the enzyme from the electrode's surface or inappropriate enzyme immobilization.

When Chitosan or PEI were used for immobilization, sensors reported the highest sensitivity: -22 mV/decade and -15 mV/decade in the linear range [-4.5, -3] log[M] respectively. Indeed, both immobilization process involved neutral polymers or less charges then previous Nafion layer. Therefore, for further optimization, enzymatic sensors with LAAO immobilized in a Chitosan polymeric matrix and LAAO crosslinked with PEI were used. Noteworthy, Pt electrodes covered with Nafion layer for *in situ* detection of Phe displayed sensitivity much higher (-80 mV/decade in the linear range [-5, -4] log[M]) than for the immobilized enzyme based sensors. Comparing the response of sensors towards Phe additions, when immobilized LAAO and non-immobilized LAAO (in situ detection) were used, it is evident that careful optimization of measurement conditions and sensor construction is necessary.

Calibration curves for Phe using sensors with LAAO immobilized in Chitosan polymeric matrix (further noted as Chitosan sensors) or crosslinked with PEI (further noted as PEI sensors) and non-immobilized LAAO are shown in Figure 5c.

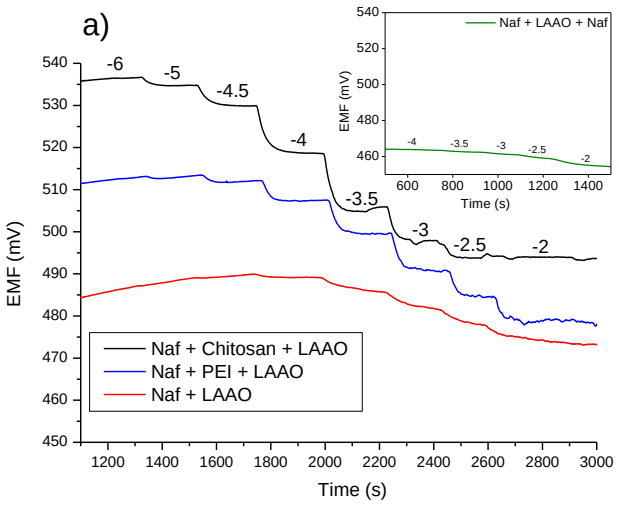

Figure 5. a) time trace and b) corresponding calibration curves for Phe additions to sensors with immobilized LAAO employing different immobilization procedures; c) Comparison of calibration curves of Phe, when immobilized LAAO and non-immobilized LAAO (in situ detection) were used.

3.3 Optimization of measurement conditions

In order to examine the influence of pH on the enzyme activity, potentiometric response of Chitosan and PEI enzymatic sensors upon additions of Phe was tested using phosphate buffer of different pH values (6.4, 7.4 and 8.4). Potentiometric response of the same sensors was also tested in *in situ* conditions, upon additions of Phe to PBS solution of particular pH value containing 0.5 mg/mL of LAAO. Sensitivities of Chitosan and PEI sensors when PBS of three different pH values was used are summarized in Table 1, whereby values in the brackets indicate linear ranges. Comparison of sensitivities for immobilized non-immobilized LAAO is also shown. The results of both experiments are in agreement and confirm that LAAO has the highest activity at pH 7.4 [21,24]. Enzyme has the lowest activity when PBS of pH 6.4 is used, whereby sensitivities are significantly reduced, even in the case of narrower linear ranges. Use of other buffers such as EPPS did not show any improvements in terms id sensitivity. Therefore, PBS of pH 7.4 was used in all further experiments.

Table 1. Sensitivity values of PEI and Chitosan sensors when PBS of different pH value is used. Comparison with in situ detection is given. Numbers in brackets display the logarithm of the linear ranges.

pH	In situ	PEI	Chitosan
6.4	-10 [-5,-4]	-0.4 [-4,-3]	-1 [-4,-3]
7.4	-80 [-5,-4]	-15 [-4.5,-3]	-22 [-4.5,-3]
8.4	-30 [-5,-4]	-15 [-4,-3]	-2 [-4,-3]

3.4 Optimization of sensor construction

Optimization of sensor construction was carried out by sequentially tuning the concentrations of immobilized LAAO, as well as the concentration of used polymer, changing the concentration of only one parameter at the time. Influence of interference species such as ascorbic acid has been taken into account by increasing the concentration of the first polymeric layer of Nafion and in that manner by changing the polymer's density of charge. Other polymers that have different density of charge such as polystyrene (PS) are also tested. All optimization procedures have been performed using the amino acid for which fabricated sensors show the highest sensitivity found in the *in situ* conditions, which in this case is Tryptophan (Trp).

a) *Optimization of enzyme concentration*

In order to examine how concentration of L-Amino Acid Oxidase (LAAO) affects sensitivity and linear range of fabricated sensor, sensors were prepared using the same procedure as explained previously but changing the concentration of LAAO. Different concentration of LAAO, namely: 0.5, 1.0, 1.5 and 2.5 mg/mL, were used for both immobilization procedures. The concentration of Chitosan/PEI was kept constant (1 % wt.), while only the concentration of LAAO was changed in order to achieve optimal conditions. Calibration curves of Chitosan sensors with different concentration of immobilized LAAO are shown in Figure 6a. In the case of sensors with LAAO immobilized in Chitosan, sensitivity increases with increasing concentration of LAAO and levels off when a concentration of 1.5 mg/mL of LAAO is reached. mg/mL of LAAO. For the concentration of 2.5 mg/mL of LAAO a reduction of the sensitivity was observed (Figure 6a Inset).

Similarly, in the case of sensors with LAAO cross-linked with PEI, whose calibration plots are shown in Figure 6b, sensitivity also increases with increasing concentration of LAAO, until a plateau is reached at 1.0 mg/mL of LAAO (Figure 6b Inset). Sensitivity values for each concentration of immobilized LAAO in the case of Chitosan/PEI sensors are summarized in Table 2.

Table 2. Sensitivity values of PEI and Chitosan sensors for different concentrations of immobilized LAAO.

C of LAAO (mg/mL)	Sensitivity (Chitosan)	Sensitivity (PEI)
0.5	-18.08	-16.83
1	-33.85	-58.49
1.5	-45.35	-54.55
2.5	-39.81	/

b) *Optimization of polymer (Chitosan/PEI) concentration*

With optimized concentration of LAAO, concentration of polymers (Chitosan, PEI) was changed in order to achieve higher sensitivity for the fabricated sensors. Concentration of polymers used was 0.5, 1.0, 1.5 and 2.5 % wt, while concentration of LAAO was 1.5 mg/mL and 1 mg/mL for Chitosan and PEI sensors, respectively. Calibration curves of Chitosan sensors with different concentration of Chitosan are shown in Figure 7a. In this case, the sensitivity also increases with increasing concentration of Chitosan until plateau is reached at a 2.5 % wt. of Chitosan (Figure 7a Inset).

Therefore, concentration of 2.5 % wt of Chitosan, that ensures highest possible sensitivity of -65.7 mV/decade, was selected.

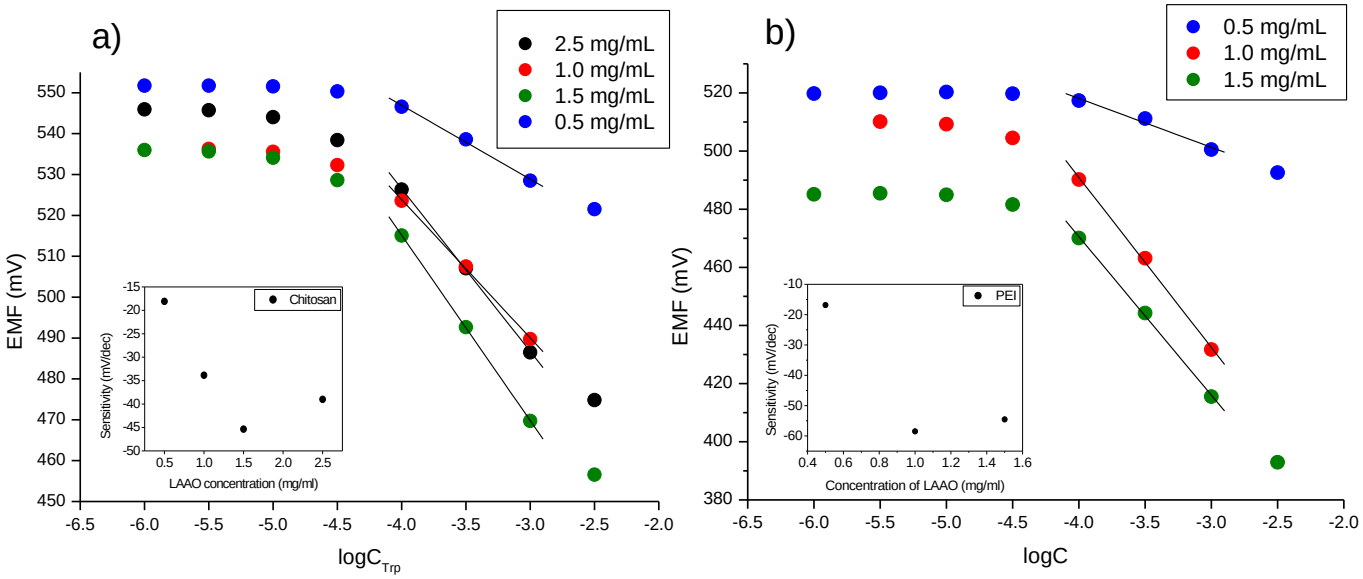

Figure 6. a) Calibration curves of Trp additions to a) Chitosan and b) PEI sensors with different concentration of immobilized LAAO. Concentration of Chitosan/PEI is in all cases 1 % wt. Insets: Sensitivity of 6a) Chitosan and 6b) PEI sensors in function of LAAO concentration.

In the case of sensors with LAAO cross-linked with PEI, increment of PEI concentration does not improve the sensitivity dramatically (Figure 7b Inset). However, concentration of 1.5 % wt of PEI is selected like the most suitable one since the sensitivity reaches the highest possible value of -66.6 mV/decade. Sensitivity values for each concentration of polymer used are summarized in Table 3.

Table 3. Sensitivity values of PEI and Chitosan sensors for different concentrations of used polymers.

C of polymer (% wt)	Sensitivity (Chitosan)	Sensitivity (PEI)
0.5	-29.06	-64.63
1	-45.34	-58.49
1.5	-58.03	-66.64
2.5	-65.68	/

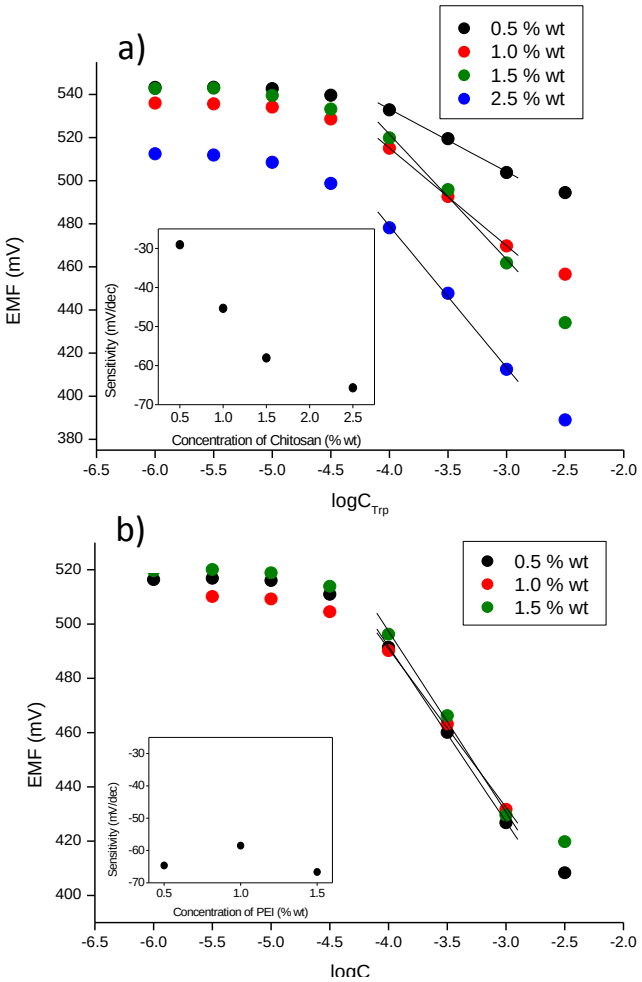

Figure 7. Calibration curves of Trp additions to a) Chitosan sensors with different concentration of Chitosan. Concentration of LAAO is in all cases 1.5 mg/mL; b) to PEI sensors with different concentration of PEI. Concentration of LAAO is in all cases 1 mg/mL.

c) Optimization of the first polymeric layer

Selectivity of fabricated sensors was assessed by monitoring the potentiometric response in presence of interfering redox species such as ascorbic acid. First polymeric layer of Nafion possess negatively charged sulfonate (SO_3^-) groups and therefore acts as a perm-selective membrane, whereby it passes neutral species such as produced H_2O_2 and repels negatively charged species such as ascorbate anion. In order to achieve highest selectivity of fabricated sensors different concentrations of Nafion (5 % wt and 20 % wt) were drop casted. In order to investigate performance of Nafion membrane, potentiometric response to 10^{-4} M ascorbate solution (which is the upper concentration in body fluids) was tested. Difference of potentials before and after addition of ascorbic acid (ΔE) is used to characterize the selectivity of the sensor and obtained results are summarized in Table 3. Both type of sensors (Chitosan and PEI) show low selectivity towards ascorbic acid when Nafion 5 % wt was used as a first polymeric layer, since the change of potential upon ascorbic acid additions is about 70 mV. When the concentration of Nafion is increased up to 20 % wt, interfering of ascorbate anion is significantly depleted, since the change of potential upon ascorbic acid additions is only 2.5 mV in the case of Chitosan sensors. This can be ascribed to the stronger repulsion between Nafion layer, that possess higher density of charge, and ascorbate anions. However, it has been observed that when concentration of Nafion is increased to 20 % wt, Chitosan sensors show a bit lower sensitivity of 45 mV/decade, compared to Chitosan sensors with 5 % wt of Nafion (Table 4). PEI sensors containing 20 % wt of Nafion show negligible sensitivity out of linear range and therefore are discarded from further optimization.

Sensors containing 5 % wt polystyrene (PS) as first polymeric layer have also been tested. In the case of Chitosan sensors, sensitivity was increased up to 72 mV/decade when PS was used, but change of potential due to ascorbic acid addition was still about 56 % of sensitivity value.

It can be concluded that sensitivity depends on density of charge of the first polymeric layer, i.e. it is improved when polymers with lower density of charge as PS are used. Therefore, in a compromise between selectivity and sensitivity, sensors containing

20 % wt of Nafion and 2.5 % of Chitosan are selected as the most suitable ones.

Table 4. Sensitivity values and potentiometric response to 10^{-4} M ascorbate solution of PEI and Chitosan sensors when type and concentration of first polymeric layer is tuned.

Sensor	Sensitivity (mV/dec)	ΔE (mV)
Naf 5 % + PEI 1.5 %	-67	75
Naf 20% + PEI 1.5%	/	/
Naf 5 % + Chitosan 2.5 %	-66	70
Naf 20% + Chitosan 2.5 %	-45	2.5
PS 5% + PEI 2.5 %	-42	25
PS 5% + Chitosan 2.5 %	-72	40

3.5. Screening of all amino acids

With a completely optimized sensor that contains Nafion 20 % wt as a first polymeric layer and 1.5 mg/mL of LAAO immobilized inside 2.5 % wt Chitosan polymeric matrix, screening of all amino acids has been done. Concentration of particular amino acid was increased from 10^{-6} M to the concentration which corresponds to amino acid's solubility. Calibration curves of all tested amino acids together with standard deviation values are shown on Figure 8. Aromatic amino acids (Phe, Tyr and Trp) show high values of sensitivity in the linear range from 10^{-4} M to 10^{-3} M (which is a range of amino acids' concentration in plasma) of -32.3, -56.1 and -45.3 mV/decade respectively. The only amino acid that shows significantly high value of sensitivity in this linear range is Cys, with sensitivity value of -67.6 mV/decade. Glu and Pro show low values of sensitivity in this linear range of -2.0 and 8.4 mV/decade. Linear ranges of Val and Arg are shifted towards higher concentrations (out of physiological range) from 10^{-3} M to 10^{-2} M and their sensitivity values are -6.0 and -15.9 mV/decade respectively.

Since amino acids Val, Arg, Pro and Glu show negligible sensitivity values in the linear range 10^{-4} M to 10^{-3} M, fabricated sensor have possible application in measurement of total amount of aromatic amino acids together with Cys. On another side, concentration of Cys could be measured with high sensitivity only by using the platinised paper electrode covered with a layer of Nafion (Figure 4). Therefore by subtracting the signal that originates from the pure Cys from the signal that comes from total amount of aromatic amino acids and Cys, concentration of only aromatic amino acids could be determined.

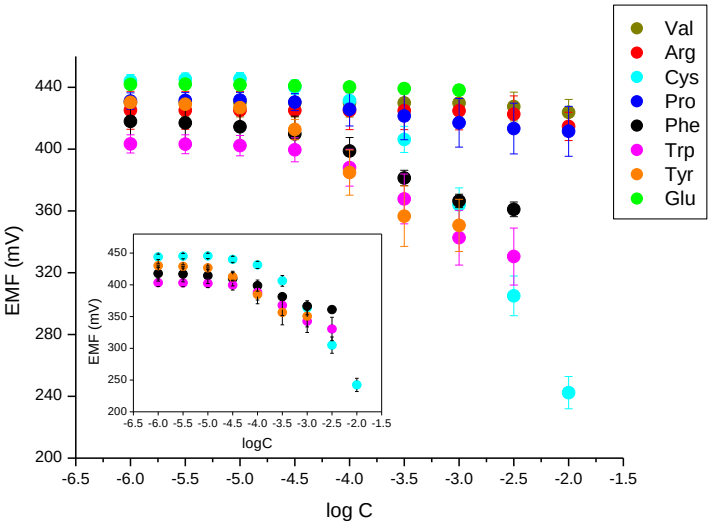

Figure 8. Calibration curves of all tested amino acids using optimized sensors.

4. CONCLUSIONS

The development of a novel paper-based enzymatic sensor for determination of amino acids was demonstrated. The potentiometric detection of the hydrogen peroxide generated by the oxidation of amino acids in presence of L-Amino Acid Oxidase (LAAO) has been proved to be a simple and effective method to detect some important amino acids. From the different methods of LAAO immobilization, incorporation into a Chitosan polymeric matrix and further addition of Nafion showed the best sensitivity and selectivity. When polymers with a lower density of charge such as PS are used instead of Nafion, the sensitivity is improved while the selectivity reduced, i.e. ascorbic acid displays significant interference.

With the fully optimized sensor, screening of amino acids with different side chain group was performed. Results show that nonpolar, aromatic amino acids (Phe, Tyr and Trp), as well as sulphur containing amino acid Cys yield highest values of sensitivity, while the rest of tested amino acids (Arg, Glu, Val and Pro) show almost negligible responses in physiological linear range. Therefore, fabricated sensor could have possible application for the measurement of total aromatic amino acid contest in different kind of fluids

(i.e. cell culture media, blood) or even for the monitoring of organic nitrogen content during the wine fermentation processes. However, these are only preliminary results and further experiments including real sample analysis should be carefully performed.

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