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Development of a novel amperometric solid-contact ion-selective sensor for the post-mortem interval determination

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Master Thesis

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Summary

This master's thesis is based on the development of an analytical measurement procedure with amperometric detection using screen-printed sensors to relate vitreous humor ions concentration to post-mortem interval. Novel amperometric solid-contact ion-selective electrodes have been developed to improve all the limitations of potentiometric ion-selective electrodes. Most important limitations of potentiometric sensors are the fixed Nernstian sensitivity depending on the charge of the target ion and the need for calibration before every analysis due to the variability of the standard potential. Moreover, amperometric sensors provide higher sensitivity, stability, reproducibility, sensor durability, faster response and lower detection limit.

Objectives

The general objective of this thesis is the development of a novel solid-contact ion-selective amperometric sensor. The master's thesis is focused on the study of different transducers and the ion-selective membrane involved in the sensor fabrication to achieve the best performance.

Specific objectives are:
- Development of a novel solid-contact ion-selective amperometric electrode.
  - Study of different transducers.
  - Study of the composition of the ion-selective membrane.
- Prediction of potassium ion concentration in artificial vitreous humor samples.

1. Introduction

This master's thesis arises from the need of some forensic institutions to have a device to perform the analysis of potassium ion concentration in biological humor vitreous samples in their laboratories. Nowadays, samples are sent to the hospital to perform the analysis and determine the post-mortem interval (PMI). The centralized potassium analysis model is the responsible for the collapse of the systems, consequently, the legal proceedings are delayed. The development of sensors that generate decentralized data makes it possible to obtain this information within a shorter period of time and will streamline all legal procedures. Moreover, PMI is very important for reconstructing the crime scene and having this information in advance can help the forensic police in its investigations.

The sensors developed in this master's thesis follow amperometry as a method of detection. This methodology is based on the measurement of the current flowing in an electrochemical cell between the working and the counter electrode under an applied constant potential between the working and the reference electrode through means of a potentiostat, as it can be observed in *Figure 1.*
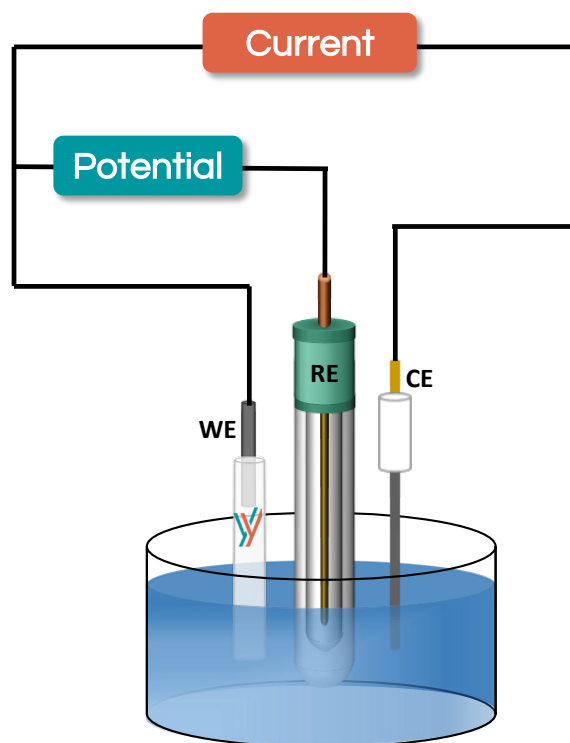
Figure 1. Schematic representation of the amperometric electrochemical principle.

Amperometric responses under potentiostatic conditions of solid-state potassium ion-selective electrodes (ISEs) have been studied in this thesis. The analytical signal recorded at the selected applied potential during potassium determination corresponds to the process occurring in the working electrode, involving either the reduction or the oxidation of the solid contact material accompanying the transfer of potassium ions across the ion-selective membrane (ISM), as shown in *Figure 2*.
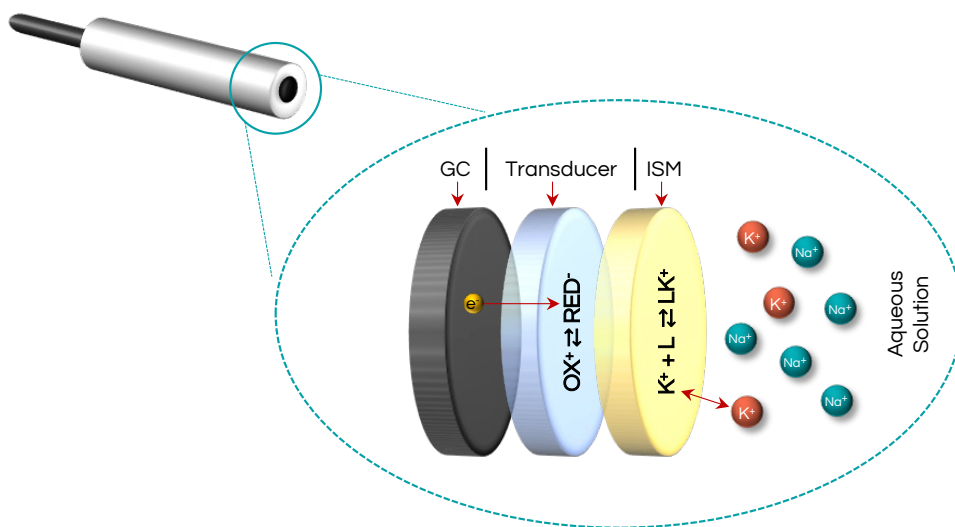
Figure 2. Schematic representation of the reduction process occurring on the electrode surface during the amperometric measurement.

Dependence of current vs. logarithm of analyte concentration is recorded, obtaining a stable response, resulting from high membrane resistance and changing membrane potential.[1] Experimental parameters as solid-contact material used, applied potential and ISM composition have been studied to obtain the best electrode performance.

Originally, ISEs were only used in potentiometric mode. More recently, solid-contact ion-selective electrodes (SC-ISEs) with thin and ultra-thin membranes and a conducting polymer (CP) layer between the membrane and the substrate, have been used as analytical tools in potentiometric, voltametric and chronoamperometric analysis[2]. An additional advantage of amperometric mode of measurements with ISEs, is the possibility to sense several ions with a single sensor containing several ionophores in the membrane[3]. However, the main benefit of chronoamperometric readout is a drastic increase on sensitivity by increasing the capacitance of the CP layer between the membrane and the substrate. In this sense, chronoamperometric mode is by far

superior to the classical potentiometric readout where the sensitivity of the ISEs is limited by the value of the Nernst factor RT/z_iF[4]. Moreover, calibration of amperometric sensors prior to use can be avoided.

The effective overpotential ($\triangle E$) under amperometric conditions with an applied potential (E_{app}) for the time where the stable current is recorded corresponds to *Equation 1.*

$$\triangle E = E_{app} - E_{ocp} = E_{app} - E^0 - s \log [K^+] \qquad \text{Equation 1}$$

E_{ocp} corresponds to the open circuit potential, E^0 is the intercept of the potentiometric measurement and s is the value of the slope in the potentiometric calibration.

The linear dependence of the current on the logarithm of the analyte concentration suggests an influence of the membrane resistance, affecting in the recorded current following the Ohm's law, $I = - \triangle E / R$. For that reason, slope is dependent on membrane resistance, thus recorded current will be higher for smaller membrane thickness and for less concentrated membrane composition. In relation with this theory, shorter time of applied potentials during amperometric readout will imply lower apparent resistance and therefore higher current involving higher slopes.[1] *Equation 2* shows the relation between potassium ion concentration and the amperometric current recorded.

$$I = \frac{E^0 - E_{appl}}{R} + \frac{s}{R} \log [K^+] \qquad \text{Equation 2}$$

The following statements can be defined:

- Current linearly increases with logarithm of analyte concentration.
- For higher negative applied potential higher current values are obtained. For positive applied potential current response depends on the standard potential.

2. Vitreous humor and post-mortem interval

Vitreous humor (VH) is a transparent, colorless and viscoelastic gel-like substance located in the space between the lens and the retina within the eye that fills the posterior cavity, as shown in *Figure 3*. VH is made up of 99 % of water, the other two main components are collagen fibrils and hyaluronic acid. VH also contains a small percentage of glycosaminoglycans (sugars), electrolytes (salts) and proteins.[5]
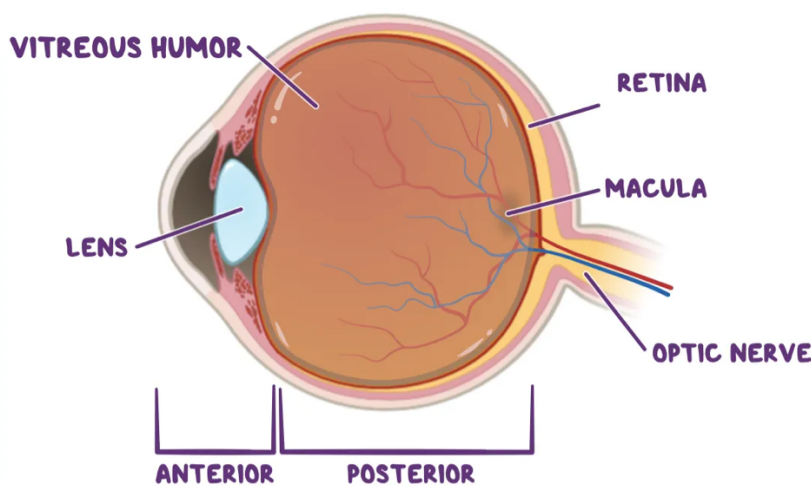
Figure 3. Eye parts scheme[6]

VH is commonly used for toxicological analysis during forensic post-mortem investigations. Potassium ion is usually analyzed for the estimation of time after death more commonly known as Post-Mortem Interval (PMI). Obtaining PMI information in the early phase of a death investigation is important for assisting the forensic police and for the decision-making prior to the autopsy.

VH offers many advantages in comparison with post-mortem serum that usually suffers hemolysis, which may interfere in the analysis. VH is a simple biological sample containing very low number of cells and therefore with small interferents since it is isolated and well-protected anatomically. In head trauma cases is less subjected to contamination and putrefaction than blood and cerebrospinal fluid. It can also be used at long periods after death since chemical changes occur at a slow rate[7].

The collection of VH is done before the autopsy by simply puncturing at the lateral aspect of the eyeball. Some analytes contained in VH are useful for different analysis:

- Glucose levels help to identify hyperglycemia.
- Potassium levels provide an estimation of the PMI.
- Sodium and chloride levels reveal dehydration or water intoxication.

Regarding VH potassium levels, an increase of its concentration is related with higher values of PMI[8]. Diffusion of potassium ions into the VH occurs after death due to the breakdown of the Na^+/K^+ pump and the loss of active membrane transport and selective membrane permeability. Potassium ions start to leave the cells of the body rapidly after death, consequently, potassium concentration in serum rises very rapidly. Contrarily, potassium levels in VH rise linearly in a time-dependent manner since the individual dies. This is due to the fact that vitreous fluids contain very few cells with similar potassium levels to other extracellular fluids. After death, leakage from surrounding retinal and choroidal cells takes place rising potassium VH levels. Potassium levels in VH are typically in the range from 5 to 40 mM.

VH potassium levels have a limited application in estimating the PMI in the first 24 h post-mortem since there are other methodologies that are working quite satisfactory as body cooling, electrical excitability of skeletal muscle or chemical excitability of iris. However, these methodologies are not used in the later PMI so the determination of potassium levels is crucial in these circumstances.[9]

Nowadays, there are many equations that have been developed to describe the linear correlation between VH potassium concentration and PMI. However, the values of the slope and the intercept made a significant difference from each other since contribution of certain conditions as ambient temperature, putrefaction, sample pre-treatment and storage are important in model parameters. Despite the extensive research and the numerous equations described, there is not a validated method that is assumed to be accurate and universal, so PMI estimation remains under study. There is still no agreement regarding the most accurate equation to describe the increase of potassium concentration and to estimate PMI. A summary of the most significant methodologies are described in *Table 1.*

Table 1. Published equations for estimating PMI from VH potassium concentration.[10]

| Authors (year)	Equation (h)	n	Max PMI (h)	Comments
Adelson et al. (1963)	PMI = 5.88 [K$^+$]–31.53	209	21	-
Sturner & Gantner (1963)	PMI = 7.14 [K$^+$]–39.1	125	104	-
Hanson et al. (1966)	PMI = 5.88 [K$^+$]–47.1	203	310	-
Coe (1969)	PMI = 6.15 [K$^+$]–38.1	145	100	A separate equation was provided for a PMI < 6 h.
Stephens & Richards (1987)	PMI = 4.20 [K$^+$]–26.65	1427	35	Outliers, drownings, SIDS, electrolyte imbalances and extreme temperatures excluded.
Madea et al. (1989)	PMI = 5.26 [K$^+$]–30.9	107	130	Cases involving elevated urea and prolonged agony were excluded.
James et al. (1997)	PMI = 4.32 [K$^+$]–18.35	100	90	Also included hypoxanthine.
Munoz et al. (2001)	PMI = 3.92 [K$^+$]–19.04	133	40	Only non-hospital cases were examined, there was a change in variables.
Zhou et al. (2007)	PMI = 5.88 [K$^+$]–32.71	62	27	-
Jashnani et al. (2010)	PMI = 1.076 [K$^+$]–2.81	120	50	Mostly included cases involving sepsis or tuberculosis.
Bortolotti et al. (2011)	PMI = 5.77[K$^+$]–13.28	164	110	-
Mihailovic et al. (2012)	PMI = 2.749 [K$^+$]–11.98	32	30	Repetitive sampling.
Siddamsetty et al. (2013)	PMI = 4.701 [K$^+$]–29.06	210	170	-
B. Zikg (2015)	$PMI = \dfrac{\ln\left((M - \frac{C_0}{M - [K^+]})\right)}{L_0 + m_A A + m_T T}$	462	409	No cases were excluded. The proposed equation includes temperature and decedent age.

3. Experimental part

3.1. Reagents, materials and equipment

High molecular weight poly(vinil chloride) (PVC), bis(2-ethylhexyl)sebacate (DOS), potassium tetrakis(pentafluorophenyl) borate (KTPFPhB) with >98 % purity, potassium ionophore I (valinomycin), tetrakis(4-chlorophenyl)borate tetradodecylammonium salt (ETH 500), sodium dodecyl sulfate (SDS), aniline, poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) 3% weight (PEDOT:PSS), solid CNTs, t-octylphenoxypolyethoxyethanol (Triton^{TM} X-100), iron (III) chloride, potassium ferrocyanide, 7,7,8,8-tetracyanoquinodimethane (TCNQ), 3-octylthiophene (OT), lithium perchlorate (LiClO$_4$), tetrabutylammonium hexafluorophosphate (Bu$_4$NPF$_6$), hydrochloric acid (HCl), ferricyanide, tetrahydrofuran (>99.9%, THF), acetonitrile (anhydrous, >99.8%), acetone and nitrobenzene (≥99.0%) are purchased from Sigma-Aldrich.

Aqueous solutions are prepared by dissolving the appropriate chloride salts (sodium and potassium analytical-grade chloride salts from Sigma-Aldrich) in deionized water (WATR-00P-25K) from Labkem.

Amperometric measurements are recorded with the instrument PalmSens controlled by PSTrace 5.8 software running on a PC. Single-junction Ag / AgCl / 3M KCl reference electrode (RE) and platinum counter electrode (CE) are used in the three-electrode cell. Glassy Carbon (GC) electrode is purchased from Metrohm. Screen-printed sensors are made of carbon ink for the CE and the working electrode (WE) and Ag / AgCl ink is used for the RE.
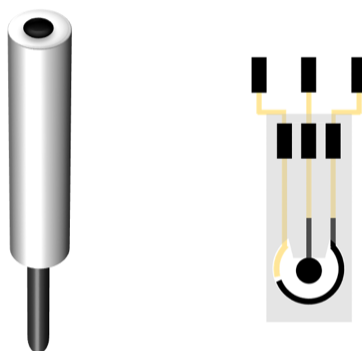
Figure 4. GC electrode (3mm diameter WE) (left) and screen-printed sensor (2mm diameter WE) (right).

3.2.   Potassium ion-selective membranes

K⁺ ISM (I) –   composition: 2.0 mg valinomycin, 0.5 mg KTPFPhB, 65.6 mg of poly(vinyl chloride) and 129.4 mg of DOS. Total 197.4 mg of membrane components are dissolved in 1 mL of THF.

K⁺ ISM (II) –   composition: 8.8 mg valinomycin, 3.5 mg KTPFPhB, 30.0 mg of poly(vinyl chloride) and 60.0 mg of DOS. Total 102.3 mg of membrane components are dissolved in 1 mL of THF.

K⁺ ISM (III) –   composition: 8.8 mg valinomycin, 2.0 mg ETH 500, 30.0 mg of poly(vinyl chloride) and 60.0 mg of DOS. Total 100.8 mg of membrane are dissolved in 1 mL of THF.

Dilution of the ISM is done by dissolving 50 µL of the corresponding membrane cocktail in 150 µL of THF.

3.3.   Transducers

The drop-casting method is used to obtain the modified GC or the screen-printed electrodes (SPEs) used for ion sensing, if not stated otherwise. GC electrodes are polished with 0.3 µm alumina powder, rinsed with water, and finally cleaned ultrasonically with water. Then for both electrode types, the intermediate layer based on the selected transducer is added and then covered with the ISM sensitive to potassium.

In this thesis, two types of WE are used (GC and SPE). First approach is performed with GC electrodes since they are used widely in the literature. However, final optimization is performed by measuring the WE of the SPE since they have already been optimized to be used with a portable device. Moreover, all the amperometric measurements have been performed by immersing the WE, platinum CE and Ag / AgCl / 3M KCl RE in the corresponding solution.

3.3.1. PEDOT:PSS

PEDOT:PSS is an organic semiconductor prepared by doping cationic poly(3,4-ethylenedioxythiophene) with poly(4-styrenesulfonate) anion. Its high electrical conductivity and good oxidation resistance makes it suitable for electromagnetic shielding and noise suppression. PEDOT:PSS is an intrinsically conducting polymer (CP) that can be coated on different substrates. In this thesis, PEDOT:PSS has been thoroughly characterized by different electrochemical techniques at different weight percentages (0.8 and 3 wt.%) and for different drop casted volumes (3, 6 and 9 µL are drop casted in successive amounts of 3 µL) on SPE.

3.3.1.1. PEDOT:PSS with CNTs

Introduction of carbon nanotubes (CNTs) between the electrode surface and the PEDOT:PSS layer increases the conductivity of the electrode and therefore the current response. CNTs have extraordinary properties due to its chemical structure as the surface-to-volume ratio and the presence of mobile electrons on the surface of the nanotubes and the capacity to promote electron transfer between heterogeneous phases acting as ion-to-electrode transducer.

Pick-and-place procedure has been followed to drop cast the CNTs layer on the SPE, using robotic machines to place surface-mount devices onto a printed circuit board. Dispersion of 1 mg/mL CNTs in 0.5% Triton^{TM} X-100 is used during the pick-and-place deposition. Moreover, a thin layer of PEDOT:PSS 3% is also added by pick-and-place. Therefore, an extra layer of 3 µL PEDOT:PSS 3% solution is drop-casted on the optimized SPE configuration, in this case with a WE of 4 mm diameter.

Finally, 8 µL in successive amounts of 2 µL of diluted K$^+$ ISM are drop casted on the modified SPE surface.

3.3.2. PANi

Polyaniline (PANi) is a CP and an organic semiconductor. In this thesis, PANi electro polymerization has been performed on a GC electrode with a solution of 0.1 M aniline in 1 M HCl by cyclic voltammetry (CV) within the potential range from +0.2 to +1 V vs. single-junction Ag / AgCl / 3 M KCl RE at 0.1 V s^{-1} scan rate. 12 CV cycles are performed. The depleted solution is then removed, and 12 CV cycles are performed again with a new aliquot of fresh aniline. Therefore, 24 CV cycles are necessary to obtain a complete modification of the electrode surface. PANi film is allowed to air-dry after electrodeposition[11]. Then, 20 µL of diluted K$^+$ ISM (I) are drop casted on the modified GC electrode surface.

3.3.3. TCNQ

TCNQ is an organic semiconductor that exhibits metallic electrical conductivity due to its high electron affinity. TCNQ forms organic charge-transfer complexes and ion-radical salts of both simple and complex composition. TCNQ is characterized by using a GC electrode, drop casting 10 µL of solution containing 2.5 mg/mL TCNQ in acetone and allowing it to dry.

TCNQ potassium salt (K(TCNQ)) has also been synthesized and characterized using a GC electrode. K(TCNQ) is prepared by drop casting 25 µL solution containing 1 mg of TCNQ dissolved in 1 mL of acetone. After solvent evaporation, electrochemical synthesis of K(TCNQ) is performed. Electrochemical reduction of solid TCNQ in the presence of potassium ions is performed in 0.1 M KCl solution by cyclic voltammetry within the potential range from +0.45 V to −0.1 V at scan rate of 20 mV s^{-1} (vs. Ag / AgCl / 3 M KCl) during 50 cycles.[12]

A mixture of 2.5 mg/mL TCNQ in acetone with K$^+$ ISM (I) (3:1) is also tested by drop casting 10 µL of this solution in the GC electrode surface and allowing it to dry.

Thereafter, 20 µL of diluted K$^+$ ISM (I) is drop casted on the three types of modified GC electrode surfaces.

3.3.4. Prussian Blue

Iron (III) hexacyanoferrate (II), mostly known as Prussian Blue (PB) can be easily coated on an electrode surface resulting in a very well-structured crystal. PB can be reduced to the colorless form (Everitt's salt) or oxidized to the yellow form (Prussian Yellow). The drop casting solution is synthesized by mixing 0.1 M potassium ferrocyanide and 0.1 M iron (III) chloride, both salts are dissolved in a solution of 0.1 M KCl and 0.01 M HCl. 6 μL of the mixture are directly drop casted on the SPE surface. The chemical reaction occurring in the electrode surface is indicated in *Equation 3*.

$$3\ K_4[Fe(CN)_6] +\ 4\ FeCl_3 \rightarrow \textbf{Fe}_4\textbf{[Fe(CN)}_6\textbf{]}_3 + 12\ KCl \qquad \text{Equation 3}$$

Previous activation before using the electrode is required: cyclic voltammetry is performed within the potential range from -0.2 to +0.6 V with a scan rate of 0.1 V s^{-1} for 13 cycles in a 0.1 M KCl solution.

Alternative electrode surface modification methodology has also been used by drop casting 6 μL of the mixture previously mentioned on the SPE surface. The electrode is dried for 20 minutes and rinsed with 0.01 M HCl solution afterwards. Cyclic voltammetry is performed within the potential range from -0.2 to +0.4 V with a scan rate of 0.04 V s^{-1} in 0.1 M KCl solution until stable voltammogram is obtained.

Electrodeposition of PB has also been tested in this thesis. For this methodology, a solution containing 2 mM of potassium ferrocyanide and iron (III) chloride in 0.1 M KCl and 0.01 M HCl media has been used for the SPE surface modification. Constant potential of +0.4 V for 50 seconds is applied. Thereafter, cyclic voltammetry within the potential range from -0.05 to +0.35 V at a scan rate of 0.4 V s^{-1} is performed until stable voltammogram is obtained.

Finally, 8 μL in successive amounts of 2 μL of diluted K$^+$ ISM are drop casted on the three types of modified SPEs surfaces.

3.3.5. POT

Poly(3-octylthiophene) (POT) is the most hydrophobic CP and due to its excellent adhesion properties, it is considered as uniquely suited to be used in combination with plasticized polymer-based ion-selective membranes. POT is recommended as ion-to-electron transducer because the POT - POT^{n+} redox couple is assumed to enhance potential stability and reproducibility.[13]

POT has been electrochemically polymerized on GC surface by performing cyclic voltammetry within the potential range from 0.0 to +1.8 V with a scan rate of 0.1 V s^{-1} for two scans in a solution containing 0.1 M 3-octylthiophene (OT) and 0.1 M $LiClO_4$ in acetonitrile. Then GC is discharged at 0.0 V for 120 s. The electrode is immersed in pure acetonitrile for 30 min to remove the electrolyte and dried at room temperature for 15 min.

3.4. Artificial samples

Due to all legal burocratic procedures that must be followed to obtain real vitreous humor samples from the IMLCFC (Institut de Medicina Legal i Ciències Forenses de Catalunya) with real potassium values indicated, it has not been possible to obtain real samples to perform potassium predictions. However, artificial samples have been prepared in the laboratory to check the performance of the electrodes.

Having into account that vitreous humor content is 99 % water, containing ions, collagen fibrils and hyaluronic acid, artificial samples have been prepared mixing NaCl and KCl solutions. Collagen fibrils and hyaluronic acid are not available in the laboratory stock, which is not a problem as theoretically they are not critical interferents for the K^+ ISM. In *Table 2*, the artificial vitreous humor samples concentrations tested are indicated.

Calibration curve for artificial vitreous humor samples prediction is obtained using two standards with potassium concentrations of 5 mM and 100 mM, respectively. Both standards contain the mean value of sodium ion concentration in vitreous humor, corresponding to 130 mM. Then, the artificial sample is measured and current response is interpolated in the calibration curve, obtaining the potassium concentration of the sample.

Calibration of the electrode before each sample measurement is needed in order to correct current irreproducibility.

Table 2. Artificial vitreous humor samples ions concentrations.

Sample	[K^+] (mM)	[Na^+] (mM)	[Cl^-] (mM)
1	10	140	150
2	20	130	150
3	30	130	160
4	8	140	148
5	12	140	152
6	35	130	165
7	40	120	160
8	26	140	166
9	24	130	154
10	32	140	172

4. Results and discussion

## 4.1. Increase of current response

Transducers and ISM compositions have been thoroughly characterized by different electrochemical techniques.

Firstly, ISM composition has been evaluated to decrease the membrane resistance and therefore increase the current response obtained, using PEDOT:PSS transducer for the first approach.

3 µL PEDOT:PSS (3% weight) are drop casted on the GC electrode surface, then two different configurations of K$^+$ ISM are tested.

1. K$^+$ ISM (I)
2. Diluted K$^+$ ISM (I) (50 µL of ISM in 150 µL of THF)

Chronoamperometric potassium calibration in water is recorded at +0.165 V constant potential with both previous configurations, the chosen potential is based on the literature.[1] *Figure 5* shows that diluted ISM provides higher current response compared to the undiluted ISM. This can be explained due to the decrease of membrane resistance when diluting it.
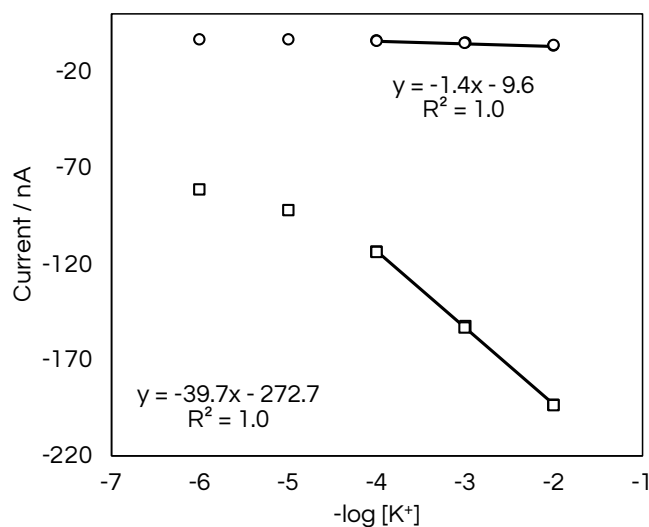
Figure 5. Potassium calibration by chronoamperometric measurement at +0.165 V. GC electrode with PEDOT:PSS as transducer. Circles indicate the response of undiluted K$^+$ ISM (I) and squares indicate the response of diluted K$^+$ ISM (I).

4.2.   Transducers

The following experiments are performed using the diluted configuration of K$^+$ ISM (I), if not stated otherwise. Different transducers are characterized by performing potassium calibrations in water along this section.

4.2.1. PEDOT: PSS

PEDOT:PSS is tested at different weight percentages (0.8 and 3.0 %). The electrodes containing higher concentration of transducer provide higher current signals. For that reason, all experiments performed to characterize PEDOT:PSS film volume and applied current are done using PEDOT:PSS transducer at 3% weight concentration.

CV of a GC containing 3 µL of PEDOT:PSS shows the oxidation and the reduction peaks at +1.0 V and -0.7 V, respectively. However, once the diluted K$^+$ ISM (I) is drop casted on the transducer surface, peaks disappear as it can be observed in *Figure 6*.
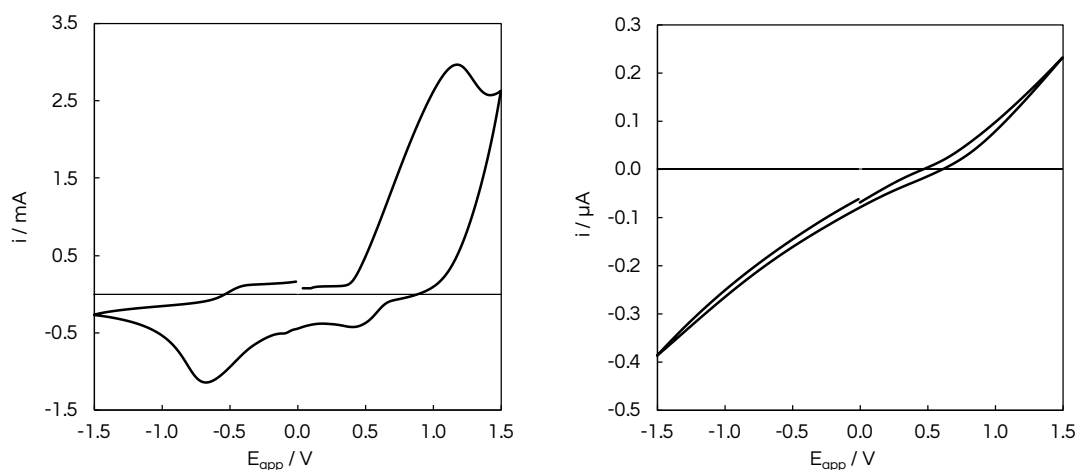

Figure 6. Observed cyclic voltammogram for 0.1 M KCl solution at 0.1 V s^{-1} scan rate. GC electrode surface modified with 3 µL PEDOT:PSS 3% (left); GC electrode surface modified with 3 µL PEDOT:PSS 3% and 10 µL K$^+$ ISM (I) (right).

The disappearance of the peaks when the ISM is added is observed with all the transducers tested in this thesis. For that reason, calibration through cyclic voltammetry technique is not possible and chronoamperometric measurements must be performed.

PEDOT:PSS drop casted volume has also been optimized. Between the different volumes, best performance and higher stability is obtained with 6 µL of PEDOT:PSS. Moreover, different applied potentials are used during potassium calibrations in water:

+0.3, +0.165, -0.2 and -0.4 V. Applied potential at -0.4 V during chronoamperometric readout is selected as the optimal potential, obtaining the higher sensitivity of 119 nA / decade with a linear range from 10^{-4} to 10^{-2} M KCl, as it can be observed in *Figure 7*.
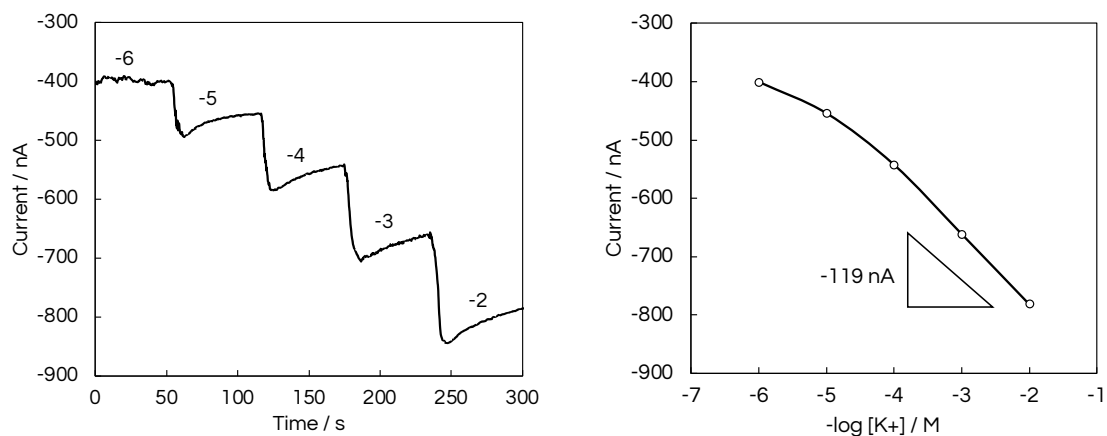
Figure 7. Chronoamperometric response for GC electrode with PEDOT:PSS transducer. Time trace (left) and corresponding calibration plot (right) upon increasing potassium concentration in water solution.

4.2.1.1. PEDOT:PSS with CNTs

Current response is improved with the addition of CNTs between the electrode surface and the PEDOT:PSS layer. SPEs containing CNTs show a sensitivity of 280 nA / decade with a linear range from 10^{-4} to 10^{-2} M KCl.

4.2.2. PANI

Aniline is electropolymerized on the GC electrode surface and PANi is characterized as a transducer. Potassium calibrations through chronoamperometric measurements at different potentials (+0.6, +0.2, -0.2 and -0.4 V) show small current response, with the higher current signal obtained for applied potential at +0.6 V with sensitivity of 69 nA / decade and linear range from 10^{-4} to 10^{-2} M KCl as shown in *Table 3*.

Table 3. Sensitivity in nA / decade during chronoamperometric potassium calibration in water at different applied potentials. LR from 10^{-4} to 10^{-2} M KCl.

Applied potential (V)	Sensitivity (nA / decade)
-0.4	39
-0.2	6
+0.2	69
+0.6	20

4.2.3. TCNQ

The three configurations mentioned in the experimental part are characterized by applying different potentials (+0.2, +0.3 and +0.4 V) during the chronoamperometric readout of potassium calibrations in water. However, currents obtained are low compared to other transducers used. The higher sensitivity is 25 nA / decade obtained using K(TCNQ) as transducer with diluted K^+ ISM (I), applying a constant potential of +0.2 V, as it can be observed in *Table 4.*

Table 4. Sensitivity in nA / decade during chronoamperometric potassium calibration in water at +0.2 V for the different configurations (see it in the Experimental Part). LR from 10^{-4} to 10^{-2} M KCl for all the configurations.

Transducer	Sensitivity (nA / decade)
TCNQ	17
K(TCNQ)	25
TCNQ / K^+ ISM	19

4.2.4. Prussian Blue

SPEs surfaces are modified with PB by using three different methodologies (see it in the Experimental Part). Higher current response is obtained with the direct drop casting of potassium ferrocyanide and iron (III) chloride solutions on the SPE and performing a CV activation prior to the electrode use. Moreover, previous activation of the electrodes through CV can be replaced by 15 min conditioning in 0.1 M KCl solution.

Among all the applied potentials during the potassium chronoamperometric calibrations, higher current response is obtained with applied potential -0.4 V, with a sensitivity of -15.8 µA / decade and a linear range from 10^{-4} to 10^{-2} M KCl. CV electrode activation is needed to avoid drifted responses during the chronoamperometric measurement. For future experiments, -0.2 V is going to be used as applied potential although smaller current is obtained to avoid applying such a high potential that can lead to reduce other undesired species, thus increasing the interferents in the obtained response.

Potassium ferrocyanide and iron (III) chloride drop casted volumes have also been optimized: 1.5 µL of each solution drop casted on the electrode surface results in a small current response and 6 µL of drop casted volume of each solution implies a high electrode current irreproducibility. For that reason, 3 µL of each solution is the optimized volume to drop cast on the SPE surface. PB is the transducer with higher current response compared to all the other used transducers. Reproducibility test is performed with a batch of five electrodes, obtaining a mean sensitivity of -4.3 ± 1.3 µA / decade and a linear range from 10^{-4} to 10^{-2} M KCl for all of them.

4.2.5. POT

Electrochemical polymerization of POT by CV within a potential range from 0.0 to +1.5 V is performed as indicated in the bibliography[3]. However, any peak of electro polymerization is obtained following this procedure. Wider potential range from 0.0 to +1.8 V is used and typical polymerization response is obtained as it can be observed in *Figure 8*.

Voltametric characterization of GC electrode containing electro polymerized POT within the potential range from 0.0 to +1.8 V is performed using a lipophilic salt (0.1 M Bu_4NPF_6) dissolved in nitrobenzene. *Figure 9* shows the cyclic voltammogram obtained where only a reduction peak is observed and no oxidation peak appears. For that reason, POT is discarded in this thesis to be used as transducer.
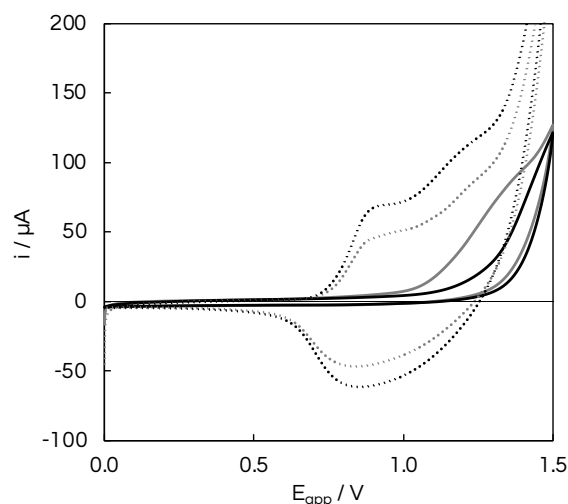
Figure 8. Electro polymerization voltammogram of POT. Grey lines correspond to the response of the first cycle and black ones to the second cycle. (−) CV within the potential range from 0.0 to +1.5V; (⋯) CV within the potential range from 0.0 to +1.8V.
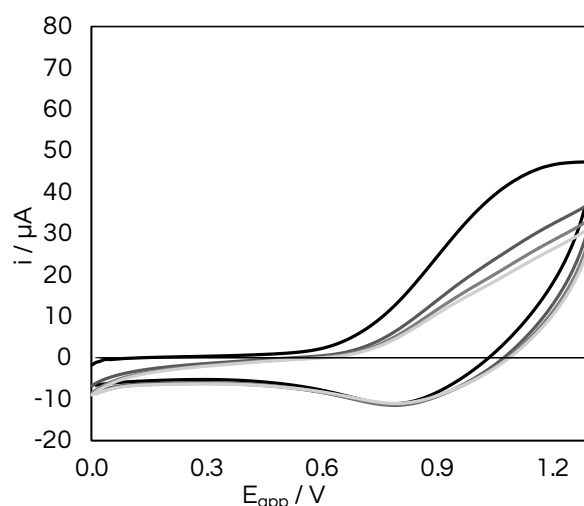
Figure 9. Observed cyclic voltammogram for 0.1 M Bu_4NPF_6 in nitrobenzene with a POT-based electrode conditioned for 24h the previous mentioned solution at 0.1 V s^{-1} scan rate. From black to grey as number of cycles increases.

4.3.   Electrodes selectivity

Sodium ions are present at higher concentration (from 120 to 140 mM) than potassium ions in vitreous humor (from 5 to 40 mM). Therefore, the selectivity of the potassium electrode in front of sodium is really important having into account that, in this analysis, sodium is the higher interferent of K^+ ISM. Potassium calibrations with sodium interferent at 0.1 M using PB optimized SPE show constant positive drift decreasing the current for 10^{-6} M KCl until stabilization, with no response for higher potassium concentrations, as it can be observed in *Figure 10.*
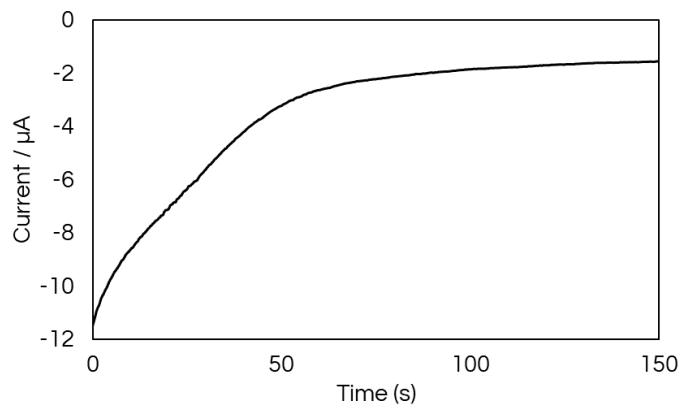
Figure 10. Chronoamperometric potassium calibration at -0.2 V using PB screen-printed optimized electrodes. *0 – 100 s*: stabilization of the electrode. *100 – 150 s*: potassium additions until 0.1 M KCl (no response observed).

Potassium ions are reversibly exchanged between PB film and aqueous solution whereas sodium ions are not. Selective ion transport has been explained in terms of the hydrated ionic radii and the channel radius in the PB lattice. The channel radius is approximately 1.6 Å and can accommodate ions as K^+, Rb^+, Cs^+ and NH_4^+ whose radii are 1.25, 1.28, 1.19 and 1.25 Å, respectively. However, it exists some electrode permeability towards sodium related to the lattice defects and the structural disorder of the microcrystalline deposit. Sodium ion was found to block the potassium ions incorporation into the PB lattice due to sodium irreversible exchange, therefore, blocking the current flow[14]. This can be the responsible of the previous observed behavior when performing potassium calibration in presence of interferent sodium ions.

Potassium calibration with interferents is performed with electrodes containing different transducers than PB. Selectivity is checked in electrodes containing PEDOT:PSS, obtaining the same chronoamperometric response observed in *Figure 10,* with the PB electrodes. For that reason, the previous theory used to explain the PB behavior in presence of sodium ions cannot be confirmed.

Selectivity test has been performed with negative applied potentials, therefore reducing the transducer and with the subsequent incorporation of potassium ions. Positive potentials have also been applied to induce the contrary effect: promote the transfer of potassium ions outside the ISM by the consequent oxidation of the transducer. Really noisy and unstable response is obtained in this experiment, without observing a clear response for potassium additions.

Chronoamperometric potassium and sodium calibrations in water are done and responses obtained are compared for SPEs containing PB and PEDOT:PSS as transducers using K^+ ISM (I). *Table 5* shows the obtained results, demonstrating that ISM does not have any selectivity towards potassium, since the same current response and linear range is obtained for sodium calibrations. Moreover, sensitivity of PB electrodes have decrease critically, without any explanation for this fact.

Table 5. K^+ and Na^+ calibrations in water through chronoamperometric measurements at -0.2 V using SPE with PEDOT:PSS or PB transducers and K^+ ISM (I).

|	PEDOT:PSS	PB
K^+ calibration	280 nA /decade	260 nA /decade
Na^+ calibration	260 nA /decade	330 nA /decade

Bibliographic references using amperometric ISEs use diluted K^+ ISM (II), containing four times more ionophore and ion-exchanger than the K^+ ISM (I). Potassium and sodium calibrations in water are performed again with K^+ ISM (II) and selectivity towards potassium is studied. *Table 6* shows the obtained results, improving the selectivity towards potassium for the PB electrode. However, PEDOT:PSS electrodes do not show a selectivity improvement.

Table 6. K^+ and Na^+ calibrations in water through chronoamperometric measurements at -0.2 V using SPEs with PEDOT:PSS or PB transducers and K^+ ISM (II).

	PEDOT:PSS	PB
K^+ calibration	220 nA /decade	800 nA /decade
Na^+ calibration	210 nA /decade	170 nA /decade

Although K^+ IMS (II) has higher selectivity towards potassium using PB transducer, when performing a potassium calibration (at 10^{-2} M NaCl solution) with a sensor that has previously been used for a sodium calibration, no response is obtained as shown *Figure 11.*
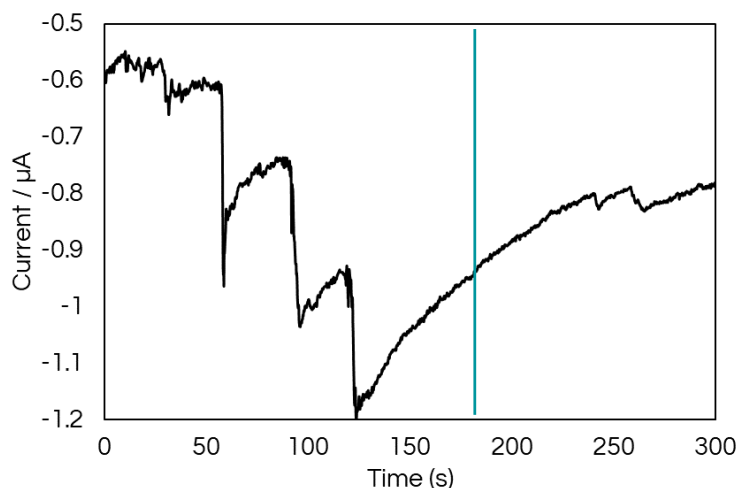
Figure 11. Sodium calibration in water (on the left of the blue line). Potassium calibration at 10^{-2} M NaCl after sodium calibration (on the right of the blue line).

The following alternatives have been tested to solve this selectivity problem:

1. Nafion® layer is drop casted between the ISM and the transducer, showing whether the problem comes from the ISM or from the transducer, since it will not directly expose the transducer to the ISM, so if there is some saturation or blocking of the transducer, theoretically Nafion® will mitigate it.

2. Direct measurement of K^+ ISM (II) without the use of any transducer involved in the redox reaction. CNTs layer is drop casted between the electrode surface and the ISM to increase the current response. This experiment is done to check the performance and selectivity of the ISM.

3. Ferricyanide is used as a mediator since it cannot incorporate potassium ions, contrarily to PEDOT:PSS and PB.

4. Measurement of the electrodes at 80 mV below the open circuit potential (OCP), in order to not apply so high potential and avoid forcing the system.

The same behavior is observed in all the previous mentioned systems, with no selectivity difference between potassium and sodium calibrations. Sensitivity for both analytes is the same, demonstrating that the ISM is not acting selectively towards potassium.

Moreover, there is no response observed when potassium calibration is performed in 0.1 M NaCl solution.

All previous experiments indicate that selectivity problem may come from the ISM and not from the transducer. The small response obtained for the potassium calibration in *Figure 11* probably comes from potassium exchanging process occurring in the ionophore. For that reason, ion-exchanger is theoretically the responsible for the lack of electrodes selectivity. To solve this problem, K^+ ISM (III) is prepared containing the same components than K^+ ISM (II) but changing the ion-exchanger to ETH 500. The use of ETH 500 lipophilic electrolyte as anionic additive is also useful to decrease the high resistance present in the membrane allowing to use it undiluted. ETH 500 is formed by a large cationic and anionic molecule, and the potential applied will force potassium to enter in the ISM. In this case, ETH 500 will not be exchanged with the solution as occurs with the potassium ion-exchanger.

Potentiometric measurement of potassium calibrations with 10^{-2} M sodium interferent using diluted K^+ ISM configurations (I) and (II) shows no potential response. However, normal behavior of a potentiometric K^+ ISM is observed with K^+ ISM (III). Amperometric measurements at 80 mV less than the OCP of electrodes containing PB or PEDOT:PSS as transducers and K^+ ISM (III) show higher selectivity, as it can be observed in *Figure 12*. With a sensitivity of 103 nA / decade and linear range from 10^{-4} to 10^{-2} M KCl.
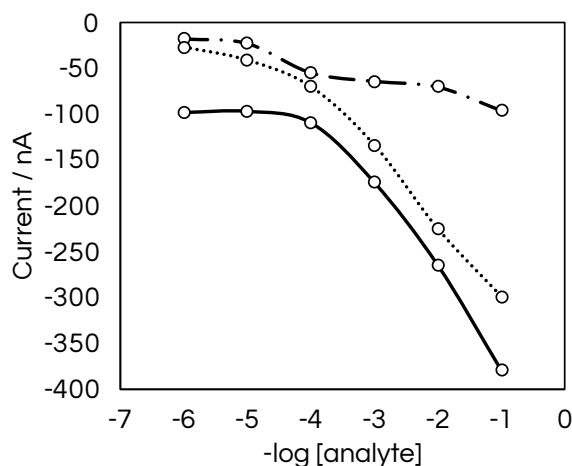
Figure 12. Chronoamperometric calibration at +0.32 V with an electrode containing PB and K^+ ISM (III). Response of different analytes in different mediums. (—) Potassium calibration in 0.1 M NaCl solution; (···) Potassium calibration in water; (-··-) Sodium calibration in water.
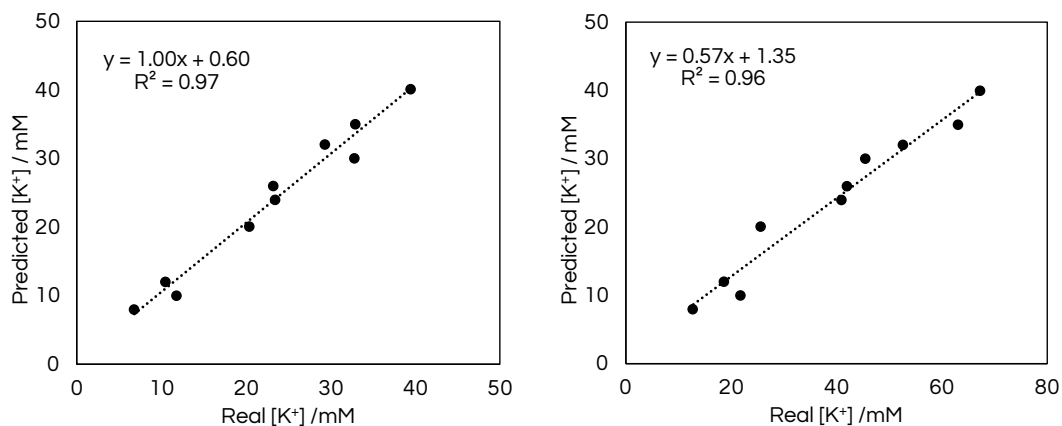
4.4.   Prediction of artificial vitreous humor samples

Prediction of potassium values of artificial vitreous humor samples are performed with the two optimized SPEs configurations, using PB or PEDOT:PSS as transducers and K^+ ISM (III). Predictions are done through chronoamperometric measurements at an applied potential of +0.32 V for both systems. Potassium calibration curve is constructed by measuring potassium standards, then interpolation of the current response obtained in the sample solution measurement provides the potassium concentration of the sample.

Although electrodes selectivity is improved using K^+ ISM (III), predictions of artificial samples do not work as expected, obtaining high prediction errors. However, a linear relation is observed when plotting real vs. predicted potassium concentration. Current instability when performing two consecutive chronoamperometric measurements, obtaining different current signals, could explain this phenomenon. To solve the current instability problem, two consecutive chronoamperometric measurements at different applied potentials are performed for the same solution. -0.2 / +0.32 V, 0.0 / +0.32 V and +0.32 / +0.42 V pair of potentials are tested, obtaining higher current difference for -0.2 and +0.32 V pair of applied potentials. However, better predictions values are obtained using +0.32 and +0.42 V.

The prediction error of artificial vitreous humor samples decreases with the use of K^+ ISM (III) (providing higher selectivity) and with the correction of the current instability (measuring at two different potentials). However, the error is high compared to the predictions performed by potentiometric measurement, as it can be observed in *Figure 13.*

Sensors with K^+ ISM (I) drop casted on a carbon path measured through potentiometry have a prediction error of 9 ± 6 % whereas for the amperometric measurement of the sensors containing PB and K^+ ISM (III), the error is 66 ± 23 %. However, linear relation plotting real vs. predicted value is observed, which indicates that current instability has not been solved by measuring at two consecutive different potentials.

Figure 13. Potentiometric prediction of artificial vitreous humor samples using K$^+$ ISM (I) on carbon electrode (left). Amperometric prediction of artificial vitreous humor samples using SPE containing PB and K$^+$ ISM (III) (right).

In the future, the electrodes developed in this thesis can be used for the determination of potassium in vitreous humor samples. However, we must firstly solve the present problem of current instability.

Deeper bibliographic research must be done in order to improve operational stability of the electrodes, and therefore, obtain reproducible and stable current response. Some alternative experiments to test are:

- The use of different transductors as polypyrrole.[1]
- The covering of the transducer with organic polymers.[15]
- The deposition of the transducer with conductive polymer matrices.[16]
- The deposition of nickel hexacyanoferrate over PB-modified SPE.[17]

5. Conclusions

The best electrode performance and higher current response are obtained with PB and PEDOT:PSS, from all conducting polymers characterized as transducers. PB provides the higher current at the beginning of the thesis, with sudden drop in the response without any explanation. Notwithstanding the current drop, PB electrodes are comparable to PEDOT:PSS.

The need to dilute the membrane to reduce the electrodes' resistance and thus increase the current response leads to a loss of selectivity of the membrane to potassium. The addition of ETH 500 as lipophilic electrolyte into the potassium ISM helps the system to reduce the high resistance and therefore undiluted configuration of the ISM can be used maintaining current response.

Although increasing the selectivity of amperometric electrodes, current instability is present when performing predictions of artificial vitreous humor samples. This may be due to the inevitable change in the conducting polymer state that leads to a higher prediction error using the new amperometric system compared to the potentiometric potassium sensor.

The use of amperometric sensors theoretically imply an improvement in analytical parameters compared to potentiometric sensors having higher sensitivity, stability, reproducibility, sensor durability, faster response and lower detection limit. However, in this thesis it was not possible to develop an electrode configuration with all the aforementioned features. Therefore, the potentiometric potassium sensor is still better in terms of prediction than the amperometric electrodes.

In conclusion, although in this thesis a large number of transducers were tested and two systems were characterized and optimized (PEDOT:PSS and PB) obtaining an amperometric electrode configuration with good performance, it is worth to work to improve the current stability in order to reduce the prediction error. In this case, the potentiometric potassium sensor could be replaced by the amperometric sensor, benefiting from all its advantages.

Future experiments to have a stable transducer providing a reproducible current response will be performed. Moreover, the introduction of different ionophores in the ISM will increase electrodes complexity, allowing the multi-analyte detection of sodium, creatinine and magnesium, for example, obtaining more relevant information from VH. This will improve PMI determination allowing the use of more complex models that integrate higher number of analytes. The measurement of real vitreous humor samples will be also an interesting experiment in this project, in order to check the theory that viscosity of the sample will not affect the sensor performance and that analytes at smaller concentration, as some proteins, are not important interferents.

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6. APPENDICES

Appendix 1. Abbreviations

CNTs – Carbon nanotubes

CP – Conducting polymer

DOS – bis(2-ethylhexyl)sebacate

E_{app} – Applied current

ETH 500 - tetrakis(4-chlorophenyl)borate tetradodecylammonium salt

GC – Glassy carbon

i – current

ISE – Ion-selective electrode

ISM – Ion-selective membrane

KTPFPhB – Potassium tetrakis (pentafluorophenyl) borate

PANI – Polyaniline

PEDOT:PSS - poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate)

PMI – Post-mortem interval

POT – Poly (3-octylthiophene)

PVC – Polyvinyl chloride

RE – Reference electrode

SC-ISE – Solid-contact Ion-selective electrode

SDS - sodium dodecyl sulfate

SPE – Screen-printed electrode

TCNQ – Tetracyanoquinodimethane

THF – Tetrahydrofuran

VH – Vitreous Humor

WE – Working electrode

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