Characterization of a new ionophore-based ion-selective electrode for the potentiometric determination of creatinine in urine

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http://dx.doi.org/10.1016/j.bios.2016.08.025

ABSTRACT

An ion-selective electrode for the highly sensitive and selective determination of creatinine in urine is presented. The enhanced recognition of creatininium cations is achieved through the use of a newly synthesized calix[4]pyrrole-based molecule, which is incorporated as an ionophore into a suitable polymeric membrane. The corresponding complex formation constants with creatininium, potassium and sodium are calculated in the polymeric membrane and confirms the strong interactions between the ionophore and the target. With this approach, the potentiometric sensor presents excellent analytical parameters with good selectivity against major components of biological fluids. Furthermore, the pH control as well as the suitable dilution factor demonstrate to be mandatory for the challenging detection in real sample. The analytical performance of the sensor is indeed tested by measuring creatinine in urine samples (N=50). The results obtained are validated against the standard colorimetric technique (Jaffé's reaction), showing that the determination can be performed in an accurate way with minimal sample manipulation. Because of the physiological relevance of the determination of creatinine and the simplicity of the approach presented, this sensor has the potential to become a powerful tool in clinical settings as well as in point of care applications.

INTRODUCTION

Creatinine is a normal metabolic byproduct generated by the body during energy production.(Burtis et al., 2012) Since its accumulation is toxic for the cells, a fine-tuned mechanism of excretion to avoid harmful levels of this substance is essential to sustain life. For this reason, creatinine is transported by bloodstream mostly to the kidneys, where it is filtered out and excreted via urination. Therefore, monitoring the levels of creatinine in both blood and urine is of outmost importance. As a key indicator of the kidney function(Israni and Kasiske, 2012), the levels of creatinine are an essential parameter to diagnose and monitor both chronic and acute kidney diseases such as infections, chronic failures, drug effects, etc. It is widely known that the concentration of creatinine in blood beyond certain threshold is a life threatening condition.(Davila and Gardner, 1987) In chronic kidney disease (a condition that affects almost 10% of the world population(Eknoyan et al., 2013)) the evolution of the patient is monitored through the blood levels of creatinine. Urinary levels (in particular 24 hour urinary excretion) are also important to evaluate the creatinine clearance. Last but not least, from an analytical standpoint the concentration of creatinine in urine is used as a normalization factor to minimize variability due to volume dilution.(Viau et al., 2004; Wagner et al., 2010) For all these reasons, the determination of creatinine is among the most common routines of the clinical laboratory.

It may then come as something of a surprise that, despite of this relevance, current methods used for the determination of creatinine show so many drawbacks(Jacobs et al., 1991) that have raised serious concerns among the medical community.(Delanghe et al., 2008) Today, the most widely used approach to determine creatinine is based on its reaction with picric acid (Jaffé reaction(Pizzolante, 1989)), a method that has been reported more than a century ago. Alternative enzyme-based colorimetric approaches are also used. However, most of these colorimetric methods are subject to errors deriving from sample color and common interferences like acetone and glucose that can perturb the color formation.(Jacobs et al., 1991)

Potentiometry is a very attractive option for the clinical lab, mostly due to its robustness and simplicity of operation and instrumentation. Nowadays, potentiometric methods using ion-selective electrodes (ISEs) are part of the routine toolkit for the determination of pH and ions in biological fluids.(Bakker and Meyerhoff, 2007) As a further proof of their

advantages, their use has been even extended to point-of-care approaches.(Novell et al., 2014) Bühlmann and co-workers introduced an ISE for the direct determination of creatinine.(Bühlmann et al., 2001) This ISE did not use any specific receptor for creatinine. Instead, it used a polymeric membrane with an ion-exchanger and chloroparaffin as the plasticizer. While creatinine could be determined in aqueous solutions, the biofouling produced by electrically neutral lipids becomes a serious limitation in any practical application. Evidently, the way to improve the selectivity of an ISE is the incorporation of an ionophore, *i.e.,* a synthetic receptor capable of binding the analyte via selective host-guest interactions. A creatinine ISEs based on this approach has been proposed some decades ago, but –once again- the performance obtained was not good enough to be applied to the analysis of real samples.(Elmosallamy, 2006; Hassan et al., 2005; Kelly et al., 1995)

Recently, we reported, for the first time, an ISE capable of determining creatinine in real samples.(Guinovart et al., 2016) This ISE hinges on the use of a phosphonate-bridged calix[4]pyrrole ionophore that shows excellent selectivity for creatinine. Here, we report the complex formation constants of the ionophore with the targets in the polymeric membrane, which confirms its superiority compared to the previously reported ionophores. We further demonstrate the influence of the pH on the detection parameters. Lastly, particular emphasis was given to the real sample analysis and on the approach to reduce the matrix interference. Dilution appeared as a crucial way to afford reliable results, so that 50 real urine samples were analyzed using the potentiometric sensor and provided values that correlated excellently with the standard Jaffé's method.

EXPERIMENTAL

Electrode preparation. Glassy carbon (GC) (Sigradur-G, Germany) rods (length: 4 cm, diameter: 0.3 cm) were introduced into a Teflon body (length: 2 cm, diameter: 0.6 cm). One end of the GC was first polished with an abrasive paper (Carbimet 600/P1200, Buehler, USA) and afterwards with alumina of different grain-size (1 and 0.03 mm, Buehler, USA).

In the supporting information it is detailed the composition of the membrane selective for creatinine and the membranes composition used for the calculation of the complex formation constants.

Instrumentation. Electromotive force (EMF) was measured at room temperature (24 ^{o}C) with a high-input impedance ($10^{15}\,\Omega$) EMF16 multichannel data acquisition device (Lawson

Laboratories, Inc. Malvern). A double junction Ag/AgCl/KCl 3 M reference electrode (type 6.0726.100, Metrohm AG) containing 1M LiOAc electrode bridge was used in all the potentiometric experiments.

The complex formation constants were obtained using a Philips IS-561 (Electrode Body ISE, Selectophore) from Sigma-Aldrich (Spain). The procedure described by Bakker and co-workers(Mi and Bakker, 1999) was followed to calculate the binding constants (see SI for more information). It has to be pointed out that for the calculation of these constants, both ion-pairing effects and membrane-internal diffusion are not taken into account.

Determinations in real samples. Urine samples were diluted with 50 mM HOAc/$Mg(OAc)_2$ at pH 3.8 in order to ensure the creatinine is in its positively charged state (creatininium cation). Samples were diluted at 1:2, 1:10 and 1:100 ratio in order to evaluate the influence of the matrix components. The results obtained were compared against the standard Jaffé method. Best predictions were obtained when samples were diluted 1:100, as it will be discussed below. Therefore, this dilution factor was used for the determination of creatinine in all the urine samples. The EMF was recorded until stable potential readings are obtained (typically less than a minute) and afterwards, the electrodes are thoroughly cleaned with double distilled water.

Under optimized conditions, the colorimetric determination of creatinine using the Jaffé's reaction reaction was carried out using 2 mL of 10 mM of picric acid in strongly basic media (250 mM NaOH) in a cuvette where 2 μL of the standard (or the sample) were added and let react for 30 minutes. The absorbance of the product was followed at 505nm. Considering the actual concentration in the cuvette (*i.e,* a dilution factor of 1:100 for standards and sample), the linear range spans from 1 μM to 100 μM of creatinine.

RESULTS AND DISCUSSION

Calix[4]pyrrole ionophore. The calix[4]pyrrole molecule used as ionophore in this study (Figure 1b) has been recently reported by our group.(Guinovart et al., 2016) There are multiple host-guest interactions that make the synthetic receptor ideal to entrap creatinine inside its cavity. These can be seen in the crystal structure calculated by X-ray of the host-guest complex featuring the ionophore and neutral creatinine (Figure 1c). The cationic species, the creatininium cation (Figure 1a), binds to the ionophore differently, making complexes with 2:1 guest:host stoichiometry. However, the binding mode within the deep

cavity of the calix[4]pyrrole has been shown to be similar. This is relevant in potentiometry since charged species are required to generate a difference of electrical potential. Thus, the evidence suggests that the same binding mode shown in the X-ray structure also operates in the ISE presented here. The ionophore was though incorporated in the polymeric matrix to build the creatinine sensor (See SI for experimental details).

Optimization of the working pH. Since the optimum response of the sensor should be correlated with the amount of creatininium ion present in the solution, the control of the pH of the solution is of utmost importance for the determination. Creatininium has a pK_a of 4.8,(Gao et al., 2013) which means that it will be the predominant species at pH values below 4.8. In order to test the effect of pH on the analytical response, experiments were performed in a cell using the sensor as working electrode in a 1 mM universal buffer containing a constant concentration of 1 mM of creatinine. The pH was initially adjusted with HCl to reach a value of 2.8, and then it was increased by successive additions of 0.1 M NaOH while recording the potentiometric response. The results of this experiment are shown in Figure 2a. The plot shows that a maximum potentiometric response was reached at pH 3.8. The response decreases steadily from pH 3.8 onwards, reflecting a response that is modulated by the distribution of species of creatinine. Figure 2b shows the calibration curves performed at pH 2.8, 3.8 and 6.5; the corresponding analytical parameters are summarized in Table 1. Whereas a Nernstian sensitivity was obtained in all three cases, the linear range and the limit of detection (LOD) have significant variations with pH. Optimum results in terms of lowest LOD and maximum linear range were obtained at pH 3.8. From the diagram of distribution of species, quick estimation of the fraction of creatininium can be calculated: 99.0%, 90.9% and 2.0 % at pH 2.8, 3.8 and 6.5, respectively. Evidently, from pH 3.8 to 2.8 the increment in the fraction of creatininium is relatively small so little changes in the potential should be expected. Also, Figure 2a shows even a slight decrease in the magnitude of the signal at higher acidities, which can be indicating some poorer detection at these very low pH values. As a matter of fact, at pH of 2.8 the LOD is increased and linear range decreased by almost one order of magnitude. However, for the purpose of this work, this is not considered extremely relevant. On the other hand, from pH 3.8 to pH 6.5 the fraction of creatininium cation decreases almost fifty times, yielding a calibration plot with a reduction of linear range and increased LOD by 2 orders of magnitude. Control experiments were performed in a similar buffer solution but without addition of creatinine

in order to assess the effect of the pH on the electrode behavior (Fig. S1a, SI). In this case, a slight reduction of the potential is also observed as the pH decreases from 3.8 to 2.8, thus confirming the effect previously observed with creatinine. All in all, optimum working pH was set at 3.8 and a 10 mM acetic acid/magnesium acetate buffer ($HOAc/Mg(OAc)_2$) adjusted at pH 3.8 was then used for the rest of the experiments.

Characterization of the analytical performance. The evaluation of the analytical performance for the determination of creatinine at the optimum pH was performed. The potentiometric time trace and the corresponding calibration curve can be seen in Figure 3. The sensor shows a Nernstian response with a sensitivity of 54.1 ± 0.6 $mV \cdot dec^{-1}$, a LOD of $10^{-6.2}$ M, a value that is far lower than any other previously reported for synthetic ionophores for creatinine,(Elmosallamy, 2006; Hassan et al., 2005) and a broad linear range, from 1 μM and up to 10 mM.

The major challenge in the potentiometric determination of creatinine is the selectivity required in the presence of interfering ions commonly found in real samples. High levels of selectivity are required due to the elevated concentrations of cations such as sodium and potassium, as well as some natural electrically neutral lipids that may interact with the organic phase (membrane) and interfere with the determination of creatinine (Bühlmann et al., 2001). In fact, this is one of the main reasons why the potentiometric sensors for creatinine reported in the literature have not found practical applications.(Bühlmann et al., 1998) For that reason, the influence of several cations typically found in biological fluids was evaluated and their respective selectivity coefficients were calculated. Table 2 shows the selectivity coefficients calculated for these cations for the sensor and the required thresholds, allowing a maximum tolerable error of 10% (see SI for error calculation), for prediction in real urine, as reported elsewhere.(Oesch et al., 1986)

As demonstrated previously (Guinovart et al., 2016), the sensor exhibits required selectivity coefficients values for Na^+ and K^+ in urine. Moreover, the newly coefficients calculated for NH_4^+, Ca^{2+}, creatine and urea are one to two orders of magnitude below the threshold values. Noteworthy, the interference from urea, one of the most abundant species in urine, is significantly reduced compared to a blank sensor (the value for the blank is shown in SI, Table S3) .

Binding constants of the ionophore with creatininium in the polymeric membrane. In order to gain more insight into the contribution of the ionophore, the formation constants ($\beta_{I:L}$, where I relates to the target analyte and L the ionophore) of the complexes between the ionophore and each separate cation in the polymeric membrane of the sensor were calculated (Table S2)(Mi and Bakker, 1999) (see SI for more detailed experimental descriptions and calculations). The values of these formation constants were log $\beta_{I:L}$= 6.60 ± 0.09 for the creatininium cation, 4.08 ± 0.13 for K^+ and 2.24 ± 0.12 for Na^+. The binding constant for the creatininium cation is 2 and 4 orders of magnitude higher than for potassium and sodium respectively.(Mi and Bakker, 1999) Remarkably, the measured binding constant for the creatininium cation in the polymeric membrane is 2 orders of magnitude higher than the one calculated in solution for the corresponding 1:1 complex, i.e. in dichloromethane.(Guinovart et al., 2016) Compared to commercial ionophores such as valinomycin (for K^+) and crown ether derivatives for (Na^+), the binding constant value seen here for creatininium is lower by several orders of magnitude. This could be related to the nature and strength of the interactions involved in the ionophore structure: typically, ionophores for alkali cations are based on strong ion-dipole interactions, whereas the present receptor employs weaker hydrogen bonds and CH-π interactions, because of the more delocalized nature of the positive charge of the creatininium cation. The higher binding constant for the creatininium cation compared to potassium and sodium is likely explained by the reduced number of available groups that can interact simultaneously with the interfering ions compared to the creatininium cation. The alkaline ions would likely be limited to establish ion-dipole interactions with the phosphonate groups. Meanwhile a superior match in functionality and size exists between the ionophore and the target creatinine cation. The binding constant values were compared to the selectivity coefficients calculated with the SSM method. The formation constant for the ionophore:K^+ complex is 2.5 orders of magnitude smaller than that for the ionophore:creatininium complex. This is in good agreement with the selectivity coefficient of -2.5 obtained with the sensor. Similarly for Na^+, the binding constants differed by 4.3 orders of magnitude which was slightly higher than the obtained selectivity coefficient of -3.7.

Analysis of real samples. The accurate potentiometric determination of creatinine in biological samples is a challenging task. Beyond the selectivity required, the sensor must be impervious to unspecific interferences –such as biofouling- due to the attachment of components of the matrix onto the membrane, which may result in significant signal

drifts(Bühlmann et al., 2001) and even permanent damage of the sensing surface of the electrode. The problem of unspecific interferences is hard to tackle because of the inherent variability of the matrix composition of real biological samples.

To illustrate this issue, the determination of creatinine in a real urine sample was studied. First, the creatinine content of the sample was determined using the standard colorimetric method (Jaffé), yielding a value of 13 mM. Thereafter, three dilutions of the sample, namely 1:2, 1:10 and 1:100, were performed using a 50 mM HAc/$Mg(Ac)_2$ pH 3.8 buffer. These dilutions were measured potentiometrically using the creatinine sensor. A 1 mM creatinine standard solution was measured in between the samples to check the integrity of the sensor. The potentiometric time trace obtained is shown in Figure 4. For comparison, this figure also shows the ideal potentiometric response that should be expected for each dilution considering the concentration determined at the beginning of the experiment. These results show that, for the most concentrated sample solution (dilution 1:2), an erratic signal is obtained. Indeed, after an initial sharp rise (well above the expected value), the signal drifts with a positive slope (approximately 7 $mV \cdot min^{-1}$, Table 3) and does not stabilize. For the second dilution (1:10), there is still a drift, but it is less pronounced (3 $mV \cdot min^{-1}$). Finally, for a dilution 1:100, the drift is reduced down to 0.4 $mV \cdot min^{-1}$ (i.e, virtually no drift) and the creatinine prediction becomes much more accurate. The signal for 1 mM creatinine standard reveals that the sensor response is not altered during the experiments. These results are summarized in Table 3, where the creatinine prediction for each dilution is presented and compared with the reference value. The recovery values are calculated between the ratio of the levels of creatinine obtained potentiometrically and the values obtained with the standard colorimetric method. It is clear that not all the urine samples would show the same degree of interference. However, the results show that a severe interference can be overcome with a 1:100 dilution. For this reason, this dilution factor was applied to all the urine samples.

The samples used for analyzing creatinine were obtained from both healthy and people with renal diseases. To perform the determination, the sensors were first calibrated with standard creatinine solutions, and then immersed in the diluted urine sample. In order to assess the prediction from the potentiometric sensor, a validation referee method using Jaffé's reaction(Pizzolante, 1989), (routinely used in the hospitals) was used to compare all the values obtained. Figure 5 shows all the values obtained for 50 urine samples. An

excellent linear correlation with the standard Jaffé's method, with sensitivity close to 1 and an intercept close to 0 is obtained, thus validating the results obtained.

It is important to stress once again the crucial interplay between sensitivity, selectivity and linear ranges that makes possible this determination. Indeed, while conventional selectivity coefficients against commonly found ions may suggest that a determination in a real sample is possible, unspecific interferences usually found in clinical samples may turn this determination unfeasible. Since these factors show a high sample-to-sample variation, the only way to play safe is to perform the dilution of the sample. Only when the system is sensitive enough to tolerate a dilution (in this case, 1:100) and remain operational the determination of the real samples is practical, and possible.

CONCLUSIONS

This work has characterized the use of a new ionophore-based potentiometric sensor for the accurate determination of creatinine in urine. The sensor reports excellent sensitivity, selectivity against common interfering ions and limits of detection. The binding constants of the ionophore with the creatininium, sodium and potassium ions in the polymeric membrane were determined. The values obtained further demonstrated the superiority of the ionophore compared to others already reported. The adjustment of pH and sample dilution are two key aspects for the optimum detection in real sample; *i.e.* the pH enhanced the detection parameters while the dilution reduced significantly the biofouling issue from the real samples. As a proof of the usefulness of this new creatinine potentiometric sensor, the simple and accurate determination of creatinine in 50 real urine samples has been demonstrated. Therefore, it could be expected that this tool will alleviate some of the current problems in the accurate analysis of creatinine, and can open new paths towards the diagnostic and monitoring of renal diseases. Current work is being performed for the optimization of the determination of creatinine in serum and blood. Additionally, with the development of a low-cost, solid-state potentiometric cell(Novell et al., 2014), the accurate, simple and affordable determination of creatinine in decentralized settings could become a reality.

ACKNOWLEDGMENTS

The authors thank Gobierno de España MINECO (projects CTQ2014-56295-R, CTQ2014-52974-REDC, CTQ2013-46404-R and Severo Ochoa Excellence Accreditation 2014-2018

SEV-2013-0319), FEDER funds (Project CTQ2014- 56295-R), the Ramón y Cajal Programme, FPI fellowship (BES-2011-048297) and the ICIQ Foundation for funding. The authors would also like to acknowledge the financial support from European Union, Marie Curie Grant PCIG09-GA-2011-293538 (Project FlexSens), the Fundación Recercaixa (Project SensAge) and CaixaImpulse programme (Project CreatSens).

FIGURES

Figure 1. Molecular structure of a) creatininium cation, b) calix[4]pyrrole molecule and c) X-Ray crystal structure of the calix[4]pyrrole ionophore with creatinine inside the cavity.

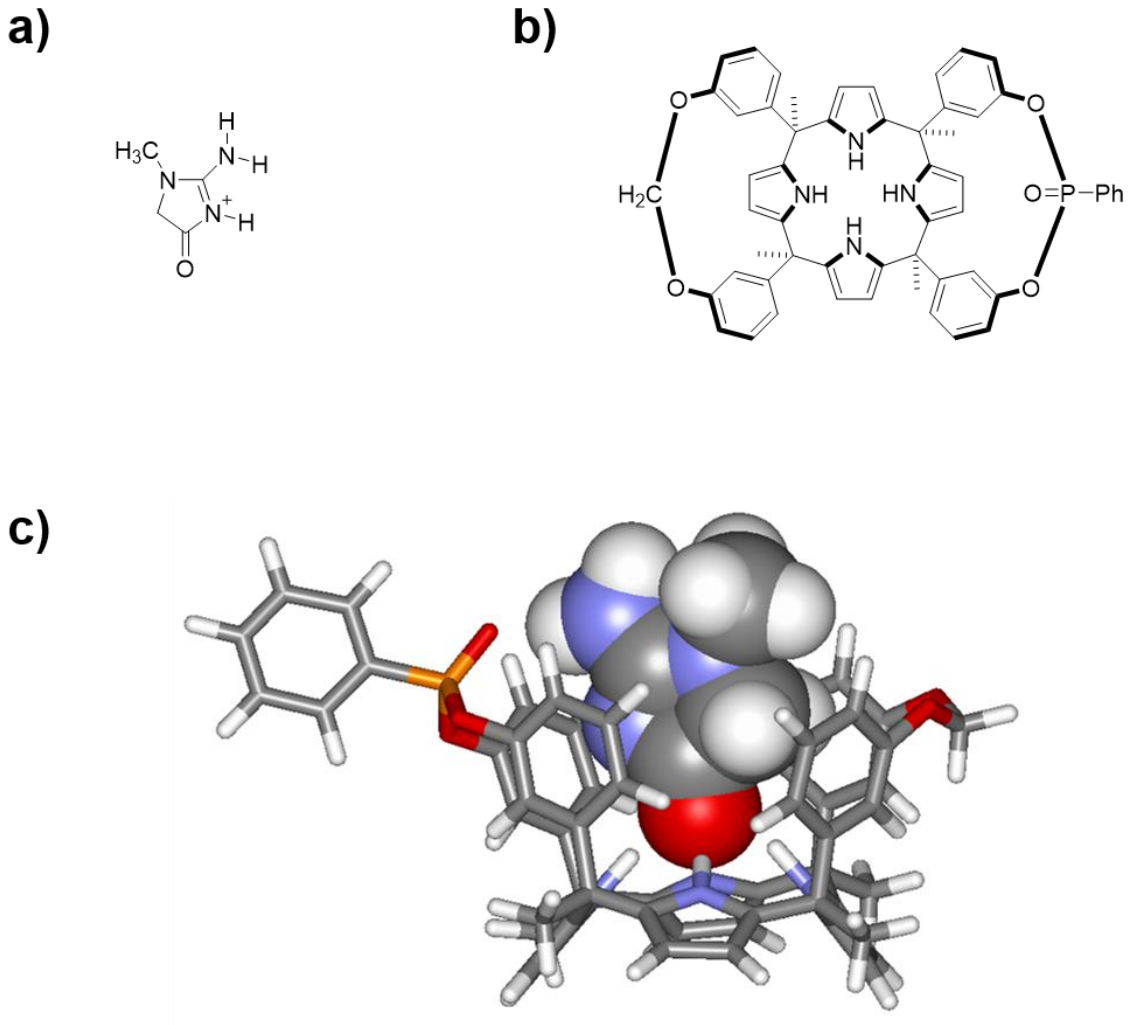


Figure 2. a) pH effect on the potentiometric response of the sensor using a 1 mM creatinine solution in 1 mM universal buffer and b) calibrations curves of creatinine at different pH: 2.8, 3.8 and 6.5.

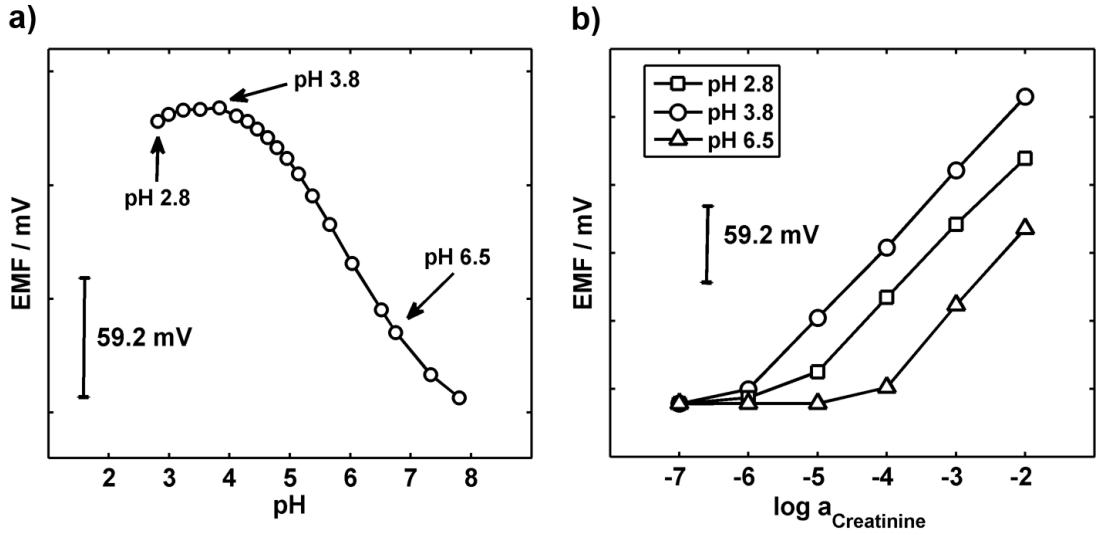


Figure 3. Potential time trace of the sensor incorporating the ionophore for different creatinine concentrations. Inset shows the corresponding calibration curve.

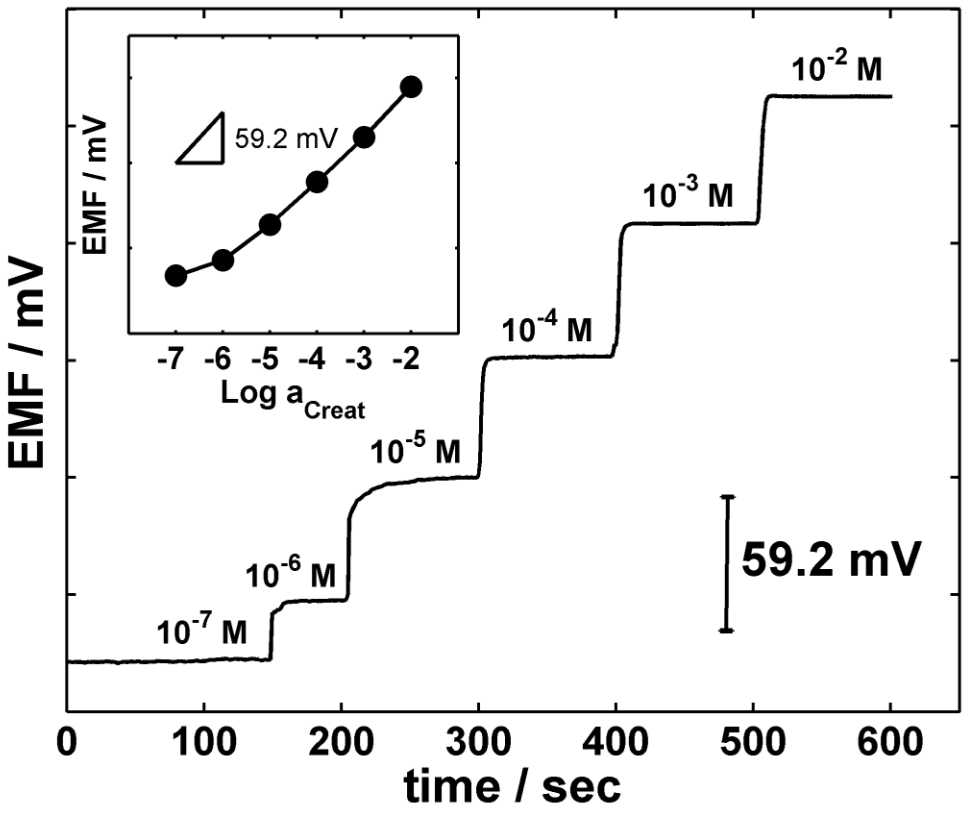


Figure 4. Expected (dashed line) and experimental (full line) potentiometric response obtained for 1:2 (a), 1:10 (b) and 1:100 (c) dilutions of urine (yellow) for the sensor. The baseline in between samples (green) corresponds to a 1 mM creatinine standard. All solutions were made with 50 mM of HOAc/$Mg(OAc)_2$ pH 3.8.

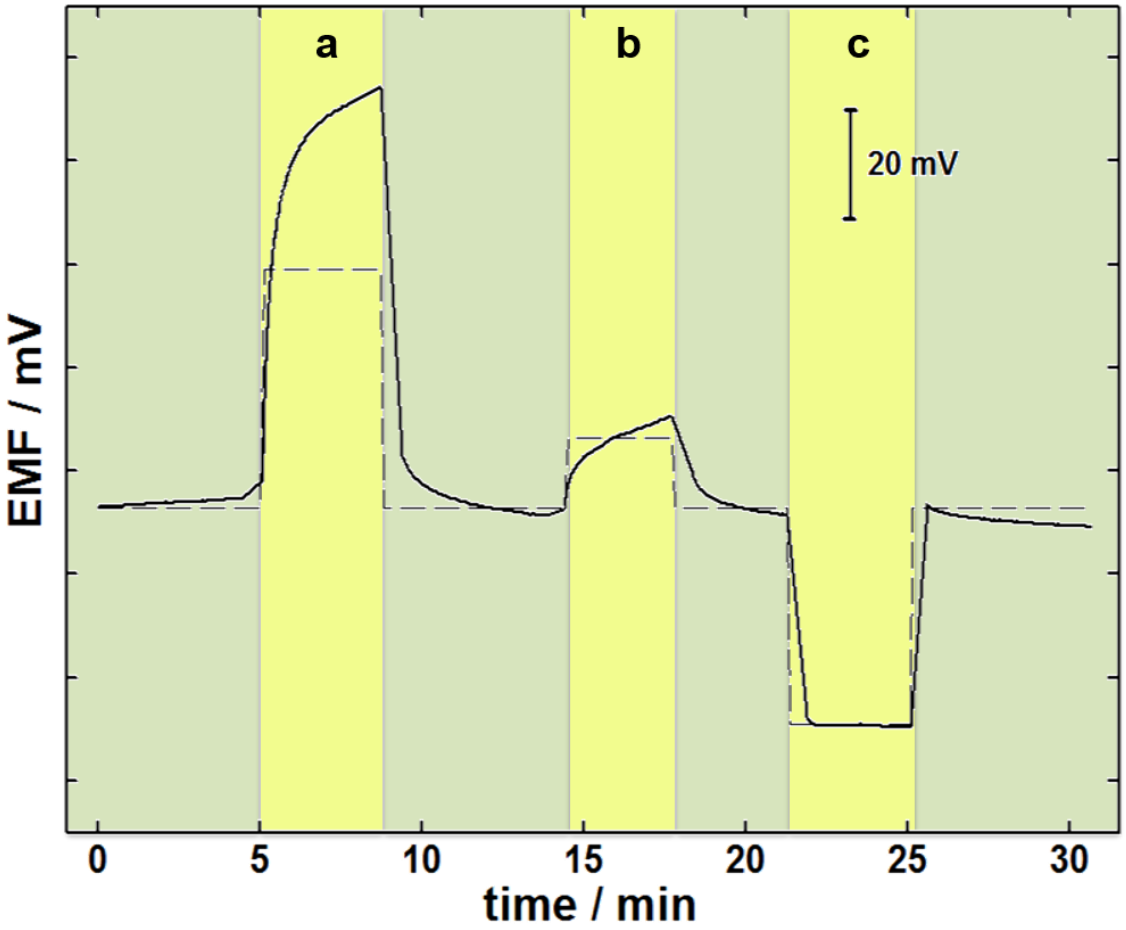
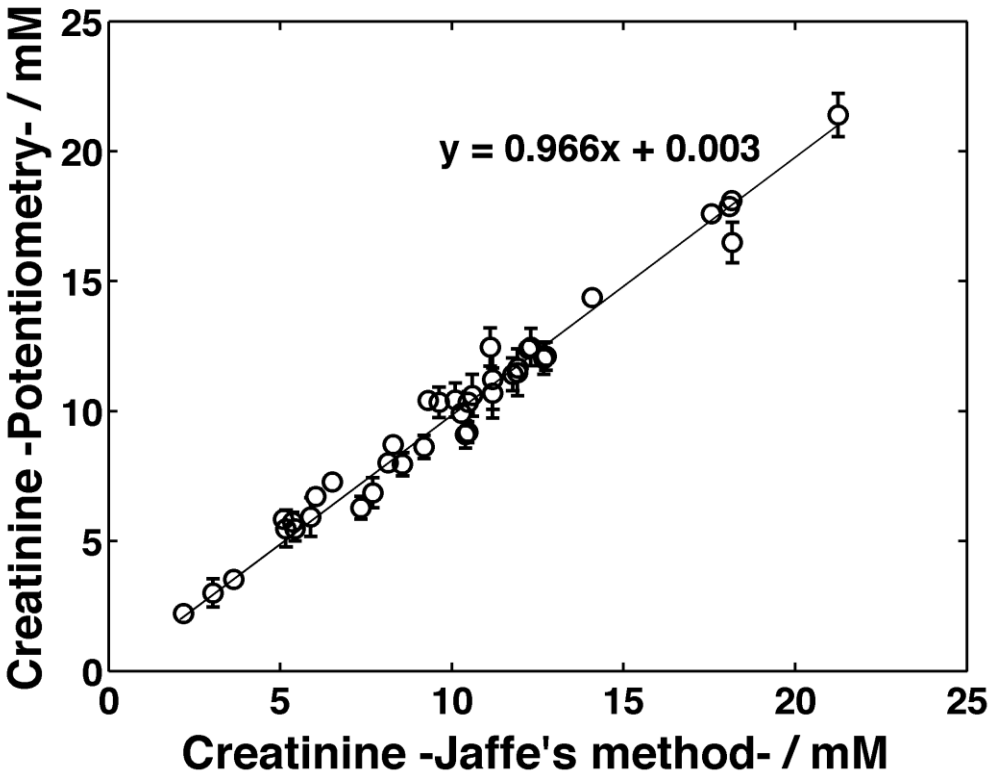


Figure 5. Creatinine values obtained in several urine samples (N=50) with the creatinine ion-selective electrode compared with the standard colorimetric standard method –Jaffé's method-.



TABLES

Table 1. Analytical parameters obtained for the sensor at different pH. The last column refers to the % of creatininium cation present in the solution at that pH.

pH	Linear Range (M)	Limit of detection (M)	Sensitivity (mV/dec^{-1})	% Creatininium cation
2.8	*10^{-5} – 10^{-2}*	*$10^{-5.5}$*	*52.5 ± 0.8*	*99.0*
3.8	*10^{-6} – 10^{-2}*	*$10^{-6.2}$*	*54.1 ± 0.6*	*90.9*
6.5	*10^{-4} – 10^{-2}*	*$10^{-4.2}$*	*58.5 ± 1.3*	*2.0*

Table 2. Selectivity coefficients (log $K_{Creatinine,J}$) for the sensor for the most relevant substances present in urine. Required threshold values for measuring in urine are also given using a maximum tolerable error (P_{IJ}) of 10%.

Analytes (J)	**Sensor** (log $K_{Creatinine,J}$)	**Required** (P_{IJ} =10%)
Urea	-4.3 ± 0.1	-2.3
Ca^{2+}	-4.8 ± 0.1	-0.5
Na^{+}	-3.7 ± 0.1	-2.6
NH_4^{+}	-2.3 ± 0.1	-1.6
K^{+}	-2.5 ± 0.1	-2.3
Creatine	-3.5 ± 0.1	-0.9

Table 3. Prediction values obtained at different dilutions of real urine. The creatinine levels are determined with the standard colorimetric method (Jaffé). The recovery is calculated using the ratio between the values obtained potentiometrically with the sensor and the standard method.

Urine dilution	***Potentiometry***	***Drift (mV·min^{-1})***	***Creatinine concentration***	***Recovery (%)***
1:2	*43 ± 6 (approx.)*	*7.0*	*13.1 ± 0.2 mM*	*330 ± 46*
1:10	*22 ± 4*	3.0		*169 ± 31*
1:100	*14 ± 1*	0.4		*107 ± 7*

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