Recognition and Sensing of Creatinine**

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Abstract: Current methods for creatinine quantification present significant drawbacks when aiming to combine accuracy, simplicity and affordability. Here, we report an unprecedented synthetic receptor, a mono-phosphonate aryl-extended calix[4]pyrrole, displaying remarkable affinity for creatinine and the creatininium cation. The receptor works by including the guests in its deep and polar aromatic cavity and establishing directional interactions in three dimensions. When incorporated into a suitable polymeric membrane, this molecule acts as an ionophore, playing the key role in a highly sensitive and selective potentiometric sensor suitable for the determination of creatinine in biological fluids such as urine or plasma in an accurate.

Considering the importance of creatinine levels with respect to human health, the current methods used for its quantification are surprisingly sub-optimal. The most widely used approach is based on the Jaffé method,[1] a method that was developed more than 100 years ago, is complex, tedious and suffers problems with interference.[2] Alternative enzyme-based assays with either colorimetric or electrochemical detection have become more common during the last decades and have overcome many issues but still show analytical and practical failings.[3] Highly accurate results are obtained using isotope dilution gas-chromatography mass-spectrometry (ID-GC-MS). Unfortunately, this approach cannot be applied in a high-traffic routine lab.[4,5] Starting in the mid-90s, supramolecular approaches for the recognition of creatinine began to appear. The designed synthetic receptors comprised an edge functionalized with convergent hydrogen bonding sites that were complementary to creatinine.[6,7,8,9,10,11]. On the other hand, ion-selective electrodes (ISEs) are among the simplest and most robust gauges of ion concentration already in used in routine clinical labs.[12] The key hurdle in the development of ISEs for relatively complex analytes like creatinine, is the availability of "ionophores", the synthetic receptors that preferentially bind the target molecule. Ionophores are responsible for the increase in ISE's sensitivity and selectivity. To the best of our knowledge there are only two examples of neutral synthetic ionophores used in potentiometric analyses of creatinine.[13,14,15] Unfortunately, the resulting ISEs did not exhibit sufficient selectivity and limit of detection for biological samples analysis.

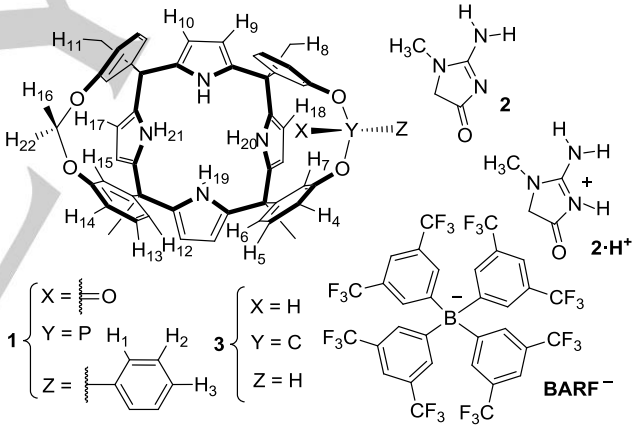

Figure 1. a) Line-drawing structures of the mono-phosphonate aryl-extended calix[4]pyrrole **1**, the reference receptor **3**, the energetically more favorable tautomer of creatinine **2** and its protonated form **2**•H^+, creatininium cation, as the BARF salt.

In this work we introduce a novel ISE suitable for the simple and accurate determination of creatinine in body fluids. This ISE hinges on a novel calix[4]pyrrole-based ionophore **1** that shows strong affinity for creatinine **2** and the creatinine cation **2**•H^+ (Figure 1). In contrast to its synthetic flat "two-dimensional" predecessors,[6,7,8,9,10,11] receptor **1** has a three dimensional shape and includes the creatinine guest **2** in its aromatic polar cavity by surrounding most of its surface. The aromatic cavity of **1** is functionalized with a single phosphonate group at its open end and four pyrrole NHs at the closed end. These polar groups offer complimentary hydrogen bonding interactions to the polar functions of the included guest. We report our detailed investigations of the binding process of **1** with both creatinine **2** and the creatininium cation **2**•H^+, in solution and in the solid

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We have investigated the use of the α,α,α,α-isomers of aryl extended calix[4]pyrroles in the complexation of polar guests in aqueous solution through a combination of hydrogen bonding, CH-π, π-π and hydrophobic interactions.[16,17] In the cone conformation, the tetra-α isomers feature a deep aromatic cavity open at one end and closed at the opposite by four pyrrole NHs. We also reported the installation of bridging polar phosphonate groups at the upper rim of phenoxy tetra-α calix[4]pyrroles.[18,19] Simple molecular modelling studies predicted that the neutral creatinine tautomer **2** was a perfect fit in terms of size and hydrogen-bonding complementarity to the polar aromatic cavity offered by mono-phosphonate-methylenebridged cavitand **1**.. Receptor **1** was prepared uneventfully starting from a previously reported tetrol aryl-extended calix[4]pyrrole.[20,21]

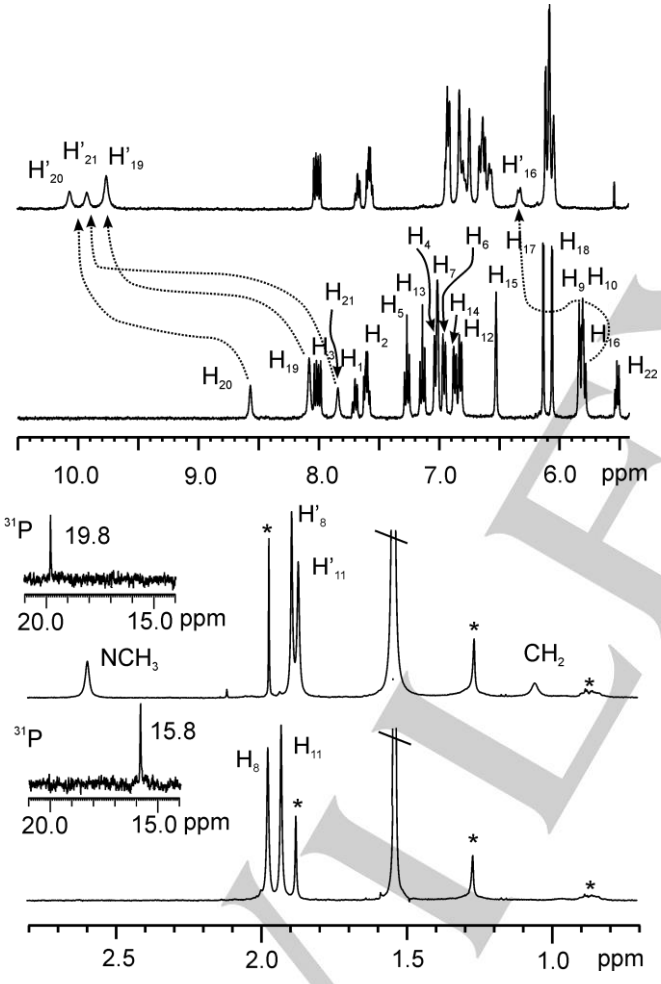

Figure 2: Selected downfield (top panel) and upfield (bottom panel) protions of the ^{1}H NMR spectra of monophosphonate cavitand **1** in CD_2Cl_2 (bottom traces) and the corresponding filtered solution obtained after a solid-liquid extraction experiment with creatinine **2** (top traces). The corresponding ^{31}P NMR spectra are shown as insets in the lower panel. * impurities

The ^{1}H-NMR spectrum of **1** in CD_2Cl_2 solution showed sharp and well resolved proton signals that were in agreement with its C_s symmetry (Figure 2, bottom traces). Taking into account the well-known low solubility of neutral creatinine **2** in organic solvents,[9] we performed solid-liquid extraction experiments. An excess of solid neutral creatinine **2** was added to a 1 mM CD_2Cl_2 solution of **1**. The resulting suspension was hand-shaken for several minutes and filtered. The filtered solution was analyzed using ^{1}H and ^{31}P-NMR spectroscopy. The ^{1}H-NMR spectrum showed a single set of proton signals for **1** with significant chemical shift changes compared to those in absence of the guest (Figure 2, top traces).

From the perspective of understanding the binding process and the complex's geometry, the most relevant shift changes occurred to the three signals belonging to the NH pyrrole protons (exhibiting a 1:1:2 integral ratio). These signals moved downfield ($\Delta\delta$ ~ 1.7-2.0 ppm) suggesting their involvement in hydrogen bonding interactions with the bound creatinine guest. Two new singlets corresponding to the methyl and methylene protons of extracted creatinine **2** appeared at δ = 2.59 ppm (CH_3) and 1.06 ppm (CH_2) (Figure 2, top right trace). When compared to the chemical shift values for neutral creatinine **2** in DMSO-d_6 solution, these signals resonate significantly further upfield (δ = 2.9 ppm for CH_3, $\Delta\delta$ ~ - 0.36 ppm and δ = 3.6 ppm for CH_2, $\Delta\delta$ ~ - 2.54 ppm). The large upfield shifts experienced by the methylene protons of the bound creatinine **2** strongly suggested its deep inclusion in the aromatic cavity of **1**. The relative integrals of the signals corresponding to **1** and **2** indicated that one equivalent of creatinine had been extracted. 2D NOESY and ROESY experiments further confirmed the proposed binding geometry (see SI, figures S2-S5). Taken together, these results show that neutral creatinine **2** forms a thermodynamically highly stable inclusion complex with receptor **1**. The oxygen atom of neutral creatinine **2** established four convergent hydrogen bonds with the pyrrole NHs and one additional hydrogen bond between the NH of the creatinine and the oxygen atom of the P=O group. The creatinine methylene hydrogen atoms were involved in CH-π interactions with two of the *meso*-aromatic walls. The significant coverage of the creatinine surface offered by the inclusion complex **2**⊂**1** represented a breakthrough with respect to receptors for this analyte. Assuming that the solubility of neutral creatinine **2** in CD_2Cl_2 is lower than 1×10^{-5} M and the exclusive formation of a 1:1 inclusion complex, we estimated a lower limit for the stability constant of K(**2**⊂**1**) = 1×10^7 M^{-1} (complex/free host ratio exceeds 100). Slow evaporation of a dichloromethane (DCM) solution yielded single crystals of the **2**⊂**1** complex suitable for X-ray diffraction analysis. Figure 3a shows the structure of the inclusion complex **2**⊂**1** in the solid state.[22] This structure is in complete agreement with the one derived from the NMR results in solution and molecular modelling studies in gas phase.

With the aim to understand the role of the phosphonate group in **1**, we performed extraction experiments of creatinine **2** with doubly methylene bridged receptor **3**, identical to cavitand **1** but lacking the phosphonate group (Figure 1). In acetone solution, receptor **1** extracted 0.40 equivalents of **2** (figure S6), whereas creatinine proton signals were not detected in control extraction experiments using reference receptor **3** or in the absence of any receptor. This result clearly supported the role of

the hydrogen bonding between the guest NH and the inwardly directed PO group in increasing the binding affinity of **1** for creatinine **2**.

In theory, to function as an ionophore in a creatinine ISE, the receptor must bind the protonated form of creatinine, **2**•H$^+$, thereby creating a charge separation across the membrane interface that culminates a voltage reading. For this reason, we evaluated the interactions of creatininium cation **2**•H$^+$ with receptor **1**, and reference bis-methylene bridged receptor **3**.

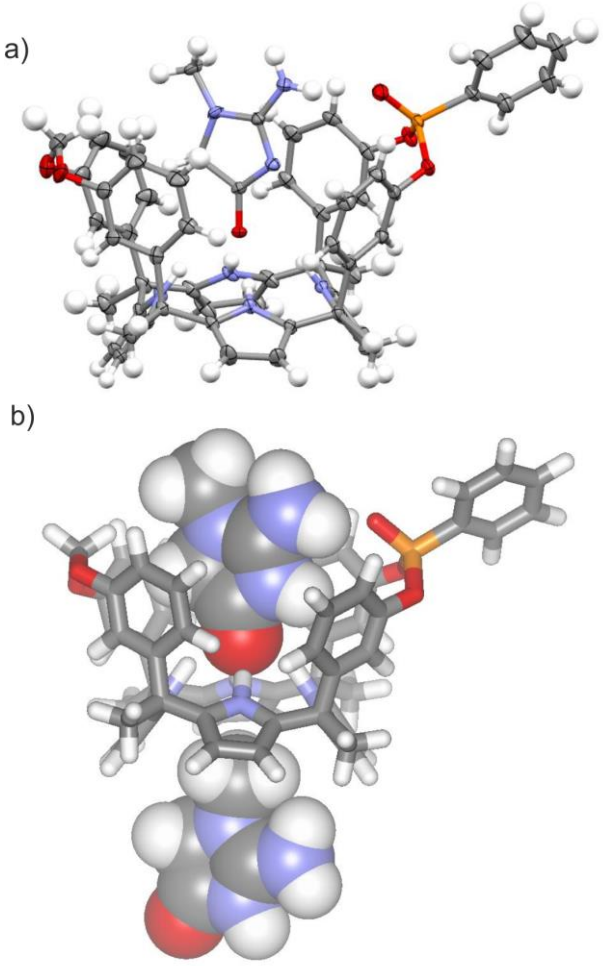

Figure 3. a) Side view of the X-ray structure of the inclusion complex **2**⊂**1**. The thermal ellipsoids are set at 50% probability level and the H atoms as spheres of 0.3 Å; b) Energy minimized structure of the putative 2:1 complex (**2**•H$^+$)$_2$⊂**1**.

Owing to the known binding affinities of calix[4]pyrroles for anions and ion-pairs, [23] we selected tetrakis(3,5-trifluoromethyl)phenylborate (BARF) (Figure 1) as a solubilizing and non-competitive counter ion for the creatininium cation. The prepared BARF$^-$•**2**•H$^+$ salt was readily soluble in DCM. We performed NMR titration experiments using receptors **1** and **3** by adding incremental amounts of BARF$^-$•**2**•H$^+$ (figure S7). The pyrrole NH protons of both receptors experienced downfield shifts. However, the extent of the shift was reduced, especially for **3**, when compared to the $\Delta\delta \approx 2$ ppm shift observed for **1** upon formation of the **2**⊂**1** complex. Based on literature precedents,[24] and our own observation in the X-ray structure of BARF$^-$•**2**•H$^+$ (figure S17), protonation of creatinine occurs at the nitrogen atom of the five-membered heterocycle (Figure 1). Protonation at this position is likely to substantially reduce the hydrogen bonding accepting properties of the adjacent carbonyl oxygen atom and might account for the reduced downfield shift of the receptors' NH protons on binding **2**•H$^+$. Interestingly, during the ^{1}H NMR titration experiment of **1** with BARF$^-$•**2**•H$^+$ we observed that the β-protons of the pyrrole rings not included in the 14-membered macrocycles (H_9 & H_{10}, Figure 1) experienced a non-monotonic chemical shift change (figure S7). This titration behavior is typically observed when complexes other than a simple 1:1 are formed. Indeed, we obtained a good fit of the titration data for H_9 & H_{10} to a theoretical binding model that assumed the formation of two complexes having 1:1 and 2:1 [**2**•H$^+$/receptor] stoichiometries. From the fit, we calculated the binding constant values for the two complexes as K(**2**•H$^+$:**1**) = 1.6×10^4 M^{-1} and K((**2**•H$^+$)$_2$:**1**) = 0.8×10^3 M^{-1}. We propose that in the 1:1 complex the **2**•H$^+$ cation is preferentially included in the deep and polar aromatic cavity of **1** resulting in a binding geometry similar to the one observed for the inclusion complex of neutral creatinine in the **2**⊂**1** complex. The interaction of the **2**•H$^+$ cation with the PO group of **1** was also supported by chemical shift changes observed in the ^{31}P NMR spectra acquired throughout the titration. Upon increasing the concentration of **2**•H$^+$, the initially formed 1:1 complex bound an additional **2**•H$^+$ cation in the shallow and electron-rich cavity defined by the pyrrole rings to produce a 2:1 complex. In short, the creatininium cation, **2**•H$^+$, showed a reduced binding affinity for receptor **1** compared to its neutral counterpart and a dual binding mode.

The titration of the reference receptor, bis-methylene bridged **3**, with BARF$^-$•**2**•H$^+$ was indicative of the exclusive formation of a 1:1 complex (K(**2**•H$^+$:**3**) = 1.0×10^3 M^{-1}). The complexation induced chemical shift changes for the methyl and methylene protons of the **2**•H$^+$ cation suggested that the creatininium cation was not included in the deep and polar aromatic cavity of **3**. Instead, the **2**•H$^+$ cation was located in the shallower and electron rich cavity defined by the pyrrole rings. A 2D ROESY experiment also supported the *exo* cation's location (figure S16). Clearly, the lack of the upper rim PO group was responsible for the selective inclusion of **2**•H$^+$ in the shallow cavity of **3** and the exclusive formation of an *exo* 1:1 complex, 2•H$^+$⊂•**3**, featuring a reduced thermodynamic stability compared to the *endo* **2**•H$^+$⊂**1**.

The working principle of the ISE is based on the partition equilibrium of **2**•H$^+$ at the membrane's phase boundary (see SI, figure S18). This is the origin of the electrical potential that ideally depends on the activity of solely creatinine ions ($a_{creatinine}$). To demonstrate the superior effect of ionophore **1** on sensor performance, we built (see SI) and tested three distinct electrodes having identical polymeric membrane composition (ion exchanger, plasticizer, etc.): (a) the "blank" sensor without embedded ionophore; (b) sensor 1, containing ionophore **1** embedded in the membrane; and (c) sensor 2 having ionophore

3 embedded. Figure 4a shows the changes in electrical potential observed on increasing the concentrations of creatininium cation **2**•H^+ for the three different sensors in buffered water solution at pH 3.4. The blank sensor showed a very limited response at low concentrations of **2**•H^+, however, at higher concentrations (above 10^{-4} M) the membrane displayed Nernstian response (59.2 mV/log $a_{creatinine}$). In the blank sensor the partition equilibrium and subsequent changes on the electromotrive force (EMF) signal are only driven by the lipophilicity of the creatininium **2**•H^+. The effect of the addition of an ionophore in the membrane is evidenced for sensors 1 and 2, in which the Nernstian response is obtained at significantly lower concentrations of **2**•H^+. For sensor 2, near ideal behavior occurs between 10 µM and 10 mM with a limit of detection (LOD) of 4.4 ± 0.6 µM. Interestingly, for sensor 1, ideal response occurs from 1 µM to 10 mM with a LOD one order of magnitude lower than for sensor 2, 0.6 ± 0.2 µM. Taken together, these results suggested that the higher affinity displayed by ionophore **1** towards the creatininium cation **2**•H^+ translated into a more sensitive ISE. Figure 4a shows the changes in EMF experienced by the three sensors when exposed to incremental concentrations of creatininium. Considering that normal levels of creatinine range from 3 to 25 mM for urine and from 0.06 to 0.42 mM for blood[25,26,27] both sensors, 1 and 2, could be adequate for analysis of real biological samples. However, the present challenge for the use of ISEs in real applications hinges on their selectivity not in their sensitivity.[12]

Some of the most severe interferences for sensing large organic cations in biological samples arise from the presence of K^+ and Na^+ that typically are vastly more prevalent than the creatininium cation.[28, 29] With this in mind, we performed selectivity studies to assess the interferences caused by the two inorganic cations (K^+ and Na^+) in the three prepared ISEs. Figure 4b depicts the EMF response of sensor 1 to increasing concentrations of creatinine and the two interferences, K^+ and Na^+. The selectivity coefficients of the three sensors for creatininium, potassium and sodium cations were calculated from the corresponding calibration curves (Figure 5a,b). In Figure 5d, the obtained values are compared to the most stringent "required selectivity coefficients" - 1.9 orders of magnitude for K^+ and -3.4 orders of magnitude for Na^+ for creatinine sensing in blood. Figure 5c depicts the same values expressed in units of concentration.

As seen in figure 5a, the blank sensor showed a selectivity pattern paralleling the lipophilicity of the cations and far from the required threshold of selectivity (Figure 5c). The introduction of ionophore **3** in the membrane (sensor 2) improved the selectivity coefficients compared to the blank sensor (Figure S19). However, the required selectivity values for a useful ISE were only achieved with ionophore **1** (sensor 1, Figure 5b).. Sensor 1 displayed an enhanced potentiometric response for the creatininium cation compared to Na^+ and K^+ and afforded selectivity coefficients values of -3.7 ± 0.1 and -2.5 ± 0.1, respectively. Compared to the blank sensor, the selectivity of sensor 1 for **2**•H^+ improved by more than one order of magnitude with respect to both interferences and exceeded the required threshold values by half order of magnitude. All in all, the analytical parameters confirmed the superiority of sensor 1 and highlighted the importance of the enhanced interaction provided by the phosphonate group present in ionophore **1**. To evaluate whether these improvements could be transferred to practical applications, we undertook the quantification of creatinine ions in real biological samples (urine and plasma) using sensor 1 (see SI).

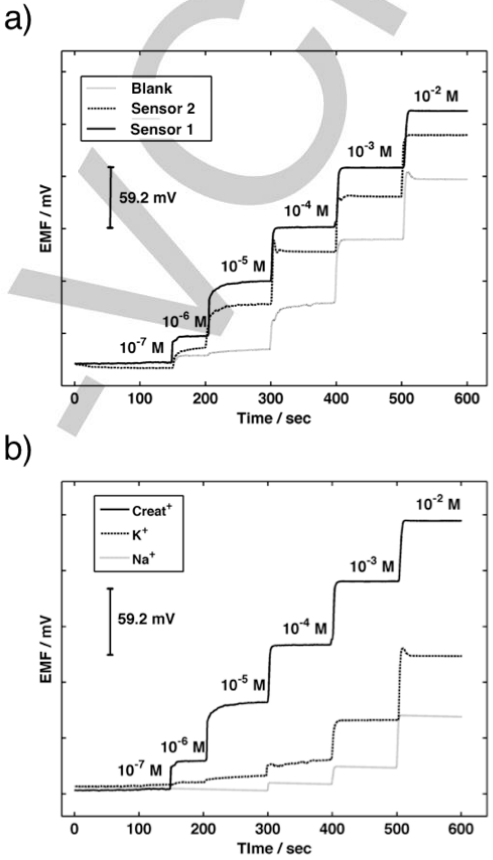

Figure 4. a) Response of the electromotive force (EMF) to incremental concentrations of creatinininium cation **2**•H^+: sensor 1 (black line), sensor 2 (dotted line) and blank sensor (grey line); b) response of the EMF of sensor 1 to the incremental concentration of several cations: $Creat^+$: creatininium cation **2**•H^+ (black line), K^+ (dotted line) and Na^+ (grey line).

Key challenge for the application of ISEs in the analysis of biological fluids derives from biofouling, i.e., the unspecific response caused by the attachment of lipophilic components onto the surface of the polymeric membrane.[30] We solved this issue of selectivity by taking advantage of the low limits of detection offered by this sensor and minimized the biofouling effect by sample dilution. Therefore, prior to analysis, urine and plasma samples were diluted 100 and 10 times respectively. We selected samples from both healthy people and patients with renal dysfunction to assess the viability of the sensor within a broad range of creatinine concentration.

After proper calibration of sensor 1, diluted biological samples were analyzed. All the measurements were compared

with the standard reference method (Jaffé's method)[2] still routinely utilized in hospitals. The results obtained are shown in Figure 6 where the values obtained with Jaffé's method are ploted vs. the values obtained with sensor 1. The data show an excellent linear correlation with slope close to 1 and intercept nearby to 0 demonstrating the validity of our methodology for the analysis of creatininium in real biological samples.

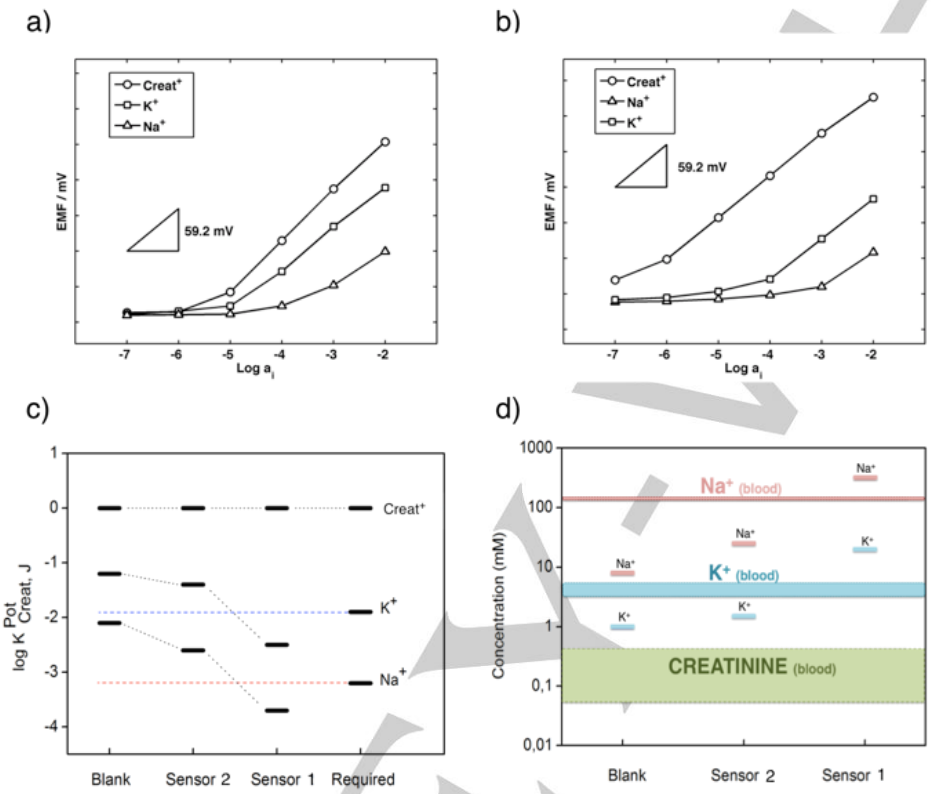

Figure 5: a) and b) Calibration curves for Creat$^+$: creatininium cation, K^+ and Na^+ obtained with the blank sensor and sensor 1, respectively. c) Representation of the selectivity coefficients in logarithmic scale and d) threshold values in concentration units, meaning the maximum allowed concentration of interference in order to be able to measure creatinine in real samples.

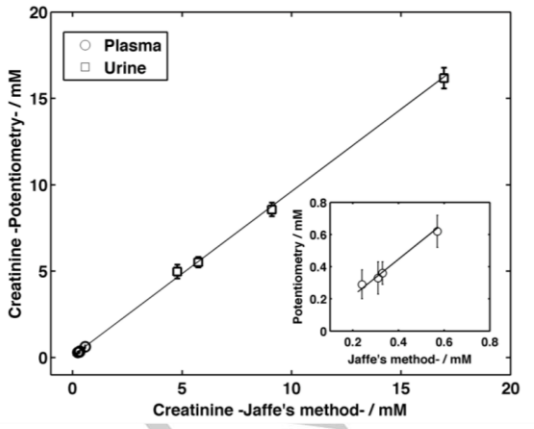

Figure 6. Plot and linear correlation of creatinine concentrations determined via potentiometric (ordinate) *vs* colorimetric (abscissa) methods for different samples of plasma and urine. Inset shows the linear regression for plasma samples only.

This work presents, for the first time, an effective sensor for the selective potentiometric detection of the creatininium cation **2**•H^+ in bodily fluids. The sensing membrane of the ISE incorporates novel receptor **1** capable of stablishing a three dimensional array of intermolecular interactions with the creatinine cation **2**•H^+ which is included in its deep and polar aromatic cavity. The binding manifold responsible for the excellent selectivity of receptor **1** towards **2**•H^+ has been studied and understood in detail. The application of receptor **1** in an ISE unveiled a potentiometric sensor with outstanding performance in terms of sensitivity and selectivity. This sensor readily detected and quantified the creatininium cation **2**•H^+ in both urine and plasma. We foresee that the described sensor device 1 will have a significant impact in current and future healthcare development.

Keywords: Cavitands •Creatinine • Ionophores • Molecular recognition • Sensors

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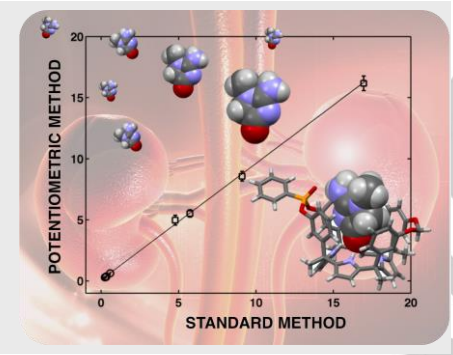

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