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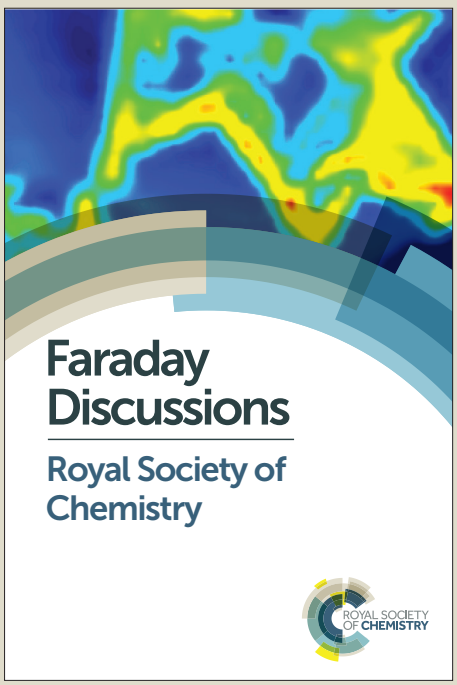

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An electrochemiluminescent-supramolecular approach to sarcosine detection for early diagnosis of prostate cancer.

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Monitoring Prostate Cancer (PCa) biomarkers is an efficient way to the early diagnosis of this disease, since it improves therapeutic success rate and suppress PCa patients mortality: for this reason a powerful analytical technique such as Electrochemiluminescence (ECL) is already used for this application, but its widespread usability is still hampered by the high cost of commercial ECL equipment. We describe an innovative approach for the selective and sensitive detection of the PCa biomarker sarcosine, obtained by a synergistic ECL-supramolecular approach, in which the free base form of sarcosine acts as co-reagent in a $Ru(bpy)_3^{2+}$-ECL process. We used magnetic micro-beads decorated with a supramolecular tetraphosphonate cavitand (**Tiiii**) for the selective capture of sarcosine hydrochloride in a complex matrix like urine. Sarcosine determination was then obtained with ECL measurements thanks to the complexation proprieties of **Tiiii**, with a protocol involving simple pH changes - to drive the capturing-releasing process of sarcosine from the receptor - and magnetic micro-beads technology. With this approach we were able to measure sarcosine in the µM-mM window, a concentration range that encompasses the diagnostic urinary value of sarcosine in healthy subject and PCa patients, respectively. These results indicate how this ECL-supramolecular approach is extremely promising for the detection of sarcosine and for PCa diagnosis and monitoring, and toward the development of portable and more affordable devices.

Introduction

Worldwide, prostate cancer (PCa) is the second most commonly diagnosed tumor in males and is responsible of more than 250.000 cancer related death.[1] An early diagnosis - when the tumour is organ confined - dramatically improves the therapeutic success rate and decreases the mortality of the PCa patients. The cancer development and progression is characterized by multiple and complex molecular events that include DNA mutations, proteins (*i.e.* Tumor Associated Antigen, TAA) overexpression, abnormal disregulation of some signalling pathways and alteration of metabolic processes (*i.e.* Warburg effect). Some of these events are under investigation to identify new tumour markers for a more accurate diagnosis and prognosis. The traditional PCa biomarker is the prostate-specific antigen (PSA). Since the era of PSA testing, a stage and grade migration has been seen with prostate cancer along with a reduction in mortality. However, PSA testing lacks specificity and reliability; in fact it can cause false positive results (serum PSA level increases also in prostatitis or benign prostatic hyperplasia). The problem of overdiagnosis may result in overtreatment with significant effects on the patient quality of life.[2] Therefore developing new PCa markers is needed for a more accurate diagnosis and moreover for both a fine selection of the therapeutic regimens and a timely monitoring of the response to therapy. Recently, *Sreekumar et al* investigated the metabolome of PCa patients to identify markers of the disease progression.[3] Specifically they identified sarcosine as a key metabolite that increases in metastatic prostate cancer and it is detectable in the urine of patients.[5,6,7] Nowadays, Electrochemiluminescence (ECL)[8,9] is the most used transduction methodology applied for the early PCa diagnosis for both the research and the commercial application.[10,11,12] In particular, ECL instrumentation based on magnetic micro-beads technology for the PSA detection is already commercialized by Roche Diagnostics Corp. (Elecsys).[13] This is the most powerful methodology for diagnostic applications, but is very expensive for a widespread use. Simple,

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inexpensive, portable sensing devices are more suitable and affordable for field operation or point-of-care testing.

Furthermore, an alternative to the immunoassay for the molecular recognition of sarcosine as a new biomarker for PCa may be provided by the supramolecular approach. Chemists are able to synthesize molecular receptors that mimic the specificity properties of biological receptor exploiting the concepts of shape recognition and binding site complementarity. The degree of sophistication reached in controlling weak host-guest interactions is such to allow the rational design of synthetic receptors according to the analyte to be detected. For this reason, synthetic receptors have a great potential in sensing and more in general in analytical chemistry. Our group reported a supramolecular cavitand for the specific recognition of sarcosine.[13,14] This tetraphosphonate cavitand shows the highest complexation constant for sarcosine hydrochloride in complex matrices, such as urine.

Here we report a sensitive methodology for the sarcosine detection in patient urine thanks to the synergistic combination between functionalized magnetic micro-beads with highly specific supramolecular recognition coupled to ECL.

Results and discussion

Analytical techniques based on ECL show several advantages over photoluminescence and chemiluminescence, in particular for (bio)sensor applications.[10,11,15] ECL has represented an important breakthrough in the analytical science; in fact, the electrochemical way to generate luminescence allows to obtain sensors with very low background and high sensitivity, good temporal and spatial resolution, robustness, versatility, and low fabrication cost.

Typically, the combination of polypyridine ruthenium complexes (in particular $Ru(bpy)_3^{2+}$, see inset in Figure 1) and amines is the most used labels/co-reactant couple in ECL applications.[16,17,18] In a typical ECL experiment in water, the energy to generate the emitting excited state comes from the homogeneous electrochemical reaction between an electrochemically-generated radical and the oxidized dye. Many amines with different structure are used as co-reactant. Although there are no strict rules matching ECL efficiency with amino groups, as a general trend, the ECL intensity increases following the order primary < secondary < tertiary amines (with tertiary amines allowing the lowest detection limits).[18,19]

In particular, tripropylamine (TPrA) is the most used sacrificial co-reactant and the main mechanism, where both dye and co-reactant are free to diffuse in the solution, is based on the so-called homogeneous *"oxidative-reduction"* co-reactant strategy.[20,21]

This mechanism involves the direct oxidation of the co-reactant, which partly undergoes a deprotonation reaction, thus forming a highly stable radical specie, able to reduce the ECL luminophore ($Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{+}$). On the other hand, the ruthenium complex can be oxidized at the electrode surface and thus can react with the $Ru(bpy)_3^{+}$ and generating the excited state $Ru(bpy)_3^{2+}*$, as it is schematized in Figure 1a.[8]

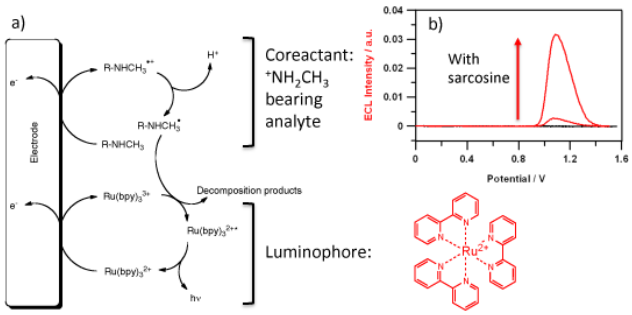


Figure 1. a) Schematic representation of ECL co-reactant "oxidative-reduction" mechanism, b) preliminary experiment: CV-ECL of $Ru(bpy)_3^{2+}$ 1 mM solution in phosphate buffer with (red line) and without (black line) 10 mM sarcosine. This experiment clearly shows that sarcosine can be used as co-reactant in the "oxidative-reduction" ECL generation.

Sarcosine, also known as *N*-methyl glycine, is a secondary amine. It shows an irreversible oxidation with a potential peak of 1 V (see SI) and thus it likely possesses the energy requirements for generating the excited state of the fluorophore. Sarcosine is therefore a good candidate to act as an ECL co-reactant.

We assesed the capability to generate ECL from sarcosine, according to the *"oxidative-reduction"* strategy (Figure 1), in a 1 mM $Ru(bpy)_3^{2+}$ phosphate buffer (PB) solution.

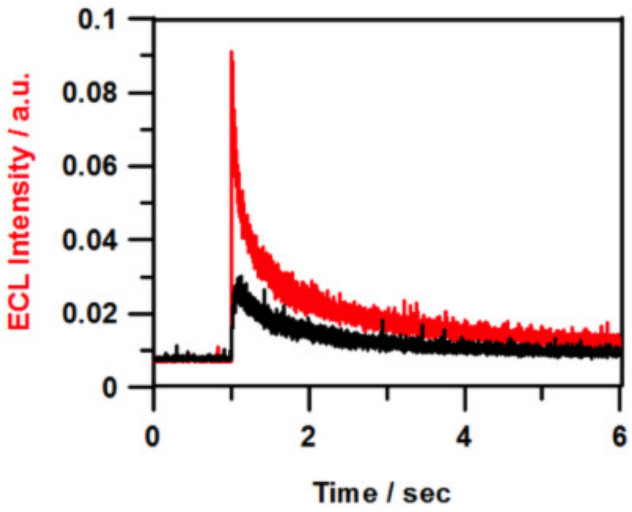


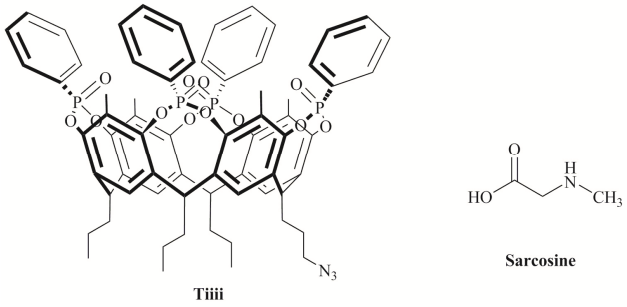
Figure 2. ECL / time transient for 10 mM of sarcosine (black line) or for 10 mM TPrA (red line) and $Ru(bpy)_3^{2+}$ 1 mM solution in phosphate buffer. PMT bias 750 V. The potential program is switched between 0V (t_1 =1 sec) and 1.4 V (t_2 = 5 sec).

In Figure 1b the ECL/potential curves before (black line) and after (red line) the addition of sarcosine are compared.

The ECL emission occurs only in presence of the sarcosine and, as expected, a maximum intensity is observed at potentials that correspond to the full oxidation of the co-reactant. In addition, the efficiency of the ECL generation for the sarcosine is 30% less than TPrA, the most efficient co-reactant reported in the literature (see comparison between the red signal and black signal in Figure 2).

Since the intensity of ECL light generated through the mechanism depicted in Figure 1 clearly depends on both the luminophore and the co-reactant concentrations - when the detection of amines is the main analytical issue - a possible analytical approach is based on measuring the ECL intensity in the presence of a constant concentration of luminophore. In such a case, the signal will depend only on the amine concentration, which becomes the limiting factor for the signal generation. This approach was largely used in literature for the quantification of amino acids, peptides,[22] nucleic acid,[23] NAD[24] and it was recently applied by Hogan and co workers in portable devices for amine detection.[25]

Supramolecular chemistry has largely been used for specific recognition of analytes, ranging from small molecules to proteins.[26,27] Here, we exploited the unique complexation properties of tetraphosphonate cavitands (**Tiiii**) toward sarcosine hydrochloride (see Scheme 1). Our target is the $^{+}NH_2CH_3$ group in the molecular structure, which is selectively recognised by **Tiiii** through the complex interplay of solvophobic, hydrogen bonding, cation-dipole and ion pairing interactions,[14] showing a very high affinity constant ($4x10^5$ M^{-1} in methanol).[28] The cavitand recognizes only the protonated sarcosine and thus, by tuning the pH, it is possible to modify the stability of the **Tiiii**-sarcosine hydrochloride complex and regulate both the capture and release of the analyte in the matrix.



Scheme 1. Chemical structure of the PCa biomarker sarcosine and **Tiiii** cavitand receptor.

To couple the supramolecular recognition properties of the cavitand with the ECL transduction and thus obtaining a sensor for sarcosine, we combined the supramolecular moieties with magnetic microbeads (MMBs). In particular, commercial MMBs with a diameter of 3.2 µm, were functionalized at the surface with **Tiiii** using a click chemistry based protocol (see the experimental part for the procedure for **Tiiii@MMB** functionalization),[29,30] to provide an efficient and robust linking between the MMB surface and the supramolecular recognition moiety. With this beads, the analytical protocol consists of three main steps: (i) *Capturing:* at acidic pH the protonated form of sarcosine is recognized and complexed by **Tiiii@MMB** (Scheme 2, inset 1); (ii) *Separation*: the beads are captured and separated from the matrix by means of a magnetic field. In this way, the sarcosine hydrochloride-**Tiiii** complex is separated from unbound amines and interferents (Scheme 2, inset 2), and finally (iii) *Release and Detection:* sarcosine is released as free base in the measuring solution by increasing the pH (Scheme 2, inset 3). The luminophore was then added, and the resulting solution deposited on disposable screen-printed electrode for ECL generation. To obtain the quantitative analyte determination, it is essential to realize the exhaustive release of sarcosine from the complex. We found that the optimal pH values for amine capturing and releasing are 5 (i.e., << sarcosine pka) and 12 (i.e., >> sarcosine pka) respectively.

The ECL intensity measured as a function of sarcosine concentration following the above mentioned methodology is shown in Figure 3. Sarcosine concentrations ranging from 50 to 3000 µM were investigated and the experimental data were analysed by the use of the following equation, describing the behaviour of ligand-receptor interaction: [31]

$$I_{ECL} = A \cdot \frac{B \cdot C_{SARCOSINE}}{1 + B \cdot C_{SARCOSINE}} \quad \text{eq. 1}$$

In equation 1, A is a constant proportional to the surface coverage of supramolecular capturing unit (**Tiiii**) on the beads surface, while B is the affinity constant of the cavitand **Tiiii** for sarcosine hydrochloride. From the best fitting (see SI) we obtained a value for the affinity constant of 2×10^3 M^{-1}.

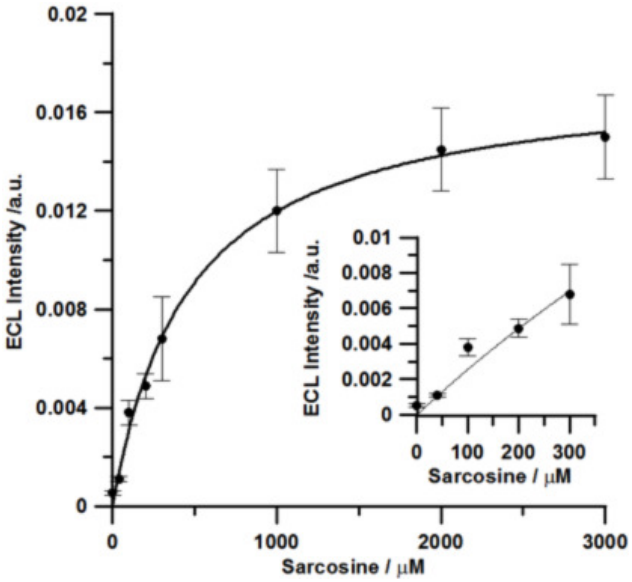


Figure 3. ECL intensity *vs* sarcosine concentration (50, 100, 200, 300, 400, 1000, 2000, 3000 µM), obtained in PB (pH 9) solution, 20 µM $Ru(bpy)_3^{2+}$ and with potential applied versus Ag/AgCl of 1.45 V. The **Tiiii@MMB** were incubate with sarcosine at pH 5 for 1 h, the **Tiiii@MMB**-sarcosine hydrochloride MMBs were finally washed three times with 0.05% Tween 20 and PB pH 5. Inset, the linear part in the calibration curve. Error bars show standard deviations (n=3).

This is significantly lower than the value reported above, obtained in solution by means of ^{1}H-NMR spectroscopy. This is a clear indication that the cavitand recognition properties are significantly affected by either its immobilization onto the bead surface, or the specific conditions used in the ECL experiment (in particular the necessary addition of a supporting electrolyte in the analytical solution), or both. The

results displayed in Figure 3 show however the great potential of the present approach for the quantitative determination of this important PCa biomarker.

The limits of detection (LOD) and quantification (LOQ) were finally determined (control plus three times and ten times standard deviation, respectively); the values obtained applying the conditions described in Figure 3 were 30 µM and 50 µM for LOD and LOQ respectively.

The LOD for the ECL sensor results significantly higher respect to LC-MS methodology, the standard approach for the detection of sarcosine in urine (1 ng ml^{-1}); however, this methodology requires a previous derivatization of sarcosine and long analytical times.[32] These drawbacks are avoided in the case of the presented protocol. Importantly, our ECL-based sensor respond to sarcosine concentrations ranging from µM to mM; it is noteworthy that this concentration window encompasses the diagnostic urinary value of sarcosine (*i.e.* sarcosine ranges from µM to mM in healthy subject and PCa patients, respectively.

Finally, the potential applicability of the proposed methodology in real samples was evaluated by assessing sarcosine in three real human urine samples, which was provided by the University Hospital of Verona according to the rules of the local ethical committee.

The urine samples were obtained by patients with diagnosed advanced metastatic disease or advanced localized disease (i.e. confirmed by imaging techniques) and previously analyzed by commercial ELISA analysis of Prostate Specific Antigen (PSA) in patients serum. As a negative control, a urine sample from a healthy donor was evaluated. The results are reported in Figure 4 and show an increase by one order of magnitude of sarcosine concentration for the PCa patient (Figure 4a, 1.91 mM and Figure 4c, 1.34 mM) compared with the healthy donor (Figure 4b, 0.27 mM).

These results indicate that this method can be applied as clinical assay to measure the urinary level of sarcosine.

Experimental

All the reagents were used without any additional purification. Tris(2,2'-bipyridine) ruthenium (II) perchlorate, $Ru(bpy)_3^{2+}$, Tripropylamine (TPrA), phosphate buffer (PB at pH 5 or pH 9), propargylamine, bathophenanthrolinedisulfonic acid disodium salt were purchased from Sigma Aldrich. Screen printed electrode C110 were purchased from DropSens®.

Tiiii[N3, CH3, Ph] (the chemical structure of the guest and the cavitand is reported in Scheme 1) was synthesized according to reported procedures.[33]

Tiiii@MMB - Magnetic microbeads functionalization

Micro-particles handling was achieved with magnetic separation and resuspending by vortexing.

200 µL of Carboxyl Magnetic Particles suspension (Sphero™ - CM-30-10, 2.5% w/v, 3.2 µm) were diluted in MES buffer (500 µL, 50 mM, pH 6.0, Triton X-100 0.01 % w/v), and washed with the same buffer solution (4 x 500 µL). The micro-beads were finally resuspended in MES buffer (160 µL), propargylamine (2 µL) was then added, followed by three separate additions of 40 µL of EDC*HCl in MES solution (50 mg/mL) performed in a 3h slot. The micro-particles were then recovered and washed (4 x 500 µL, PBS 1X, pH 7.4), and 250 µL of the final 500 µL suspension was set aside as control.

The remaining micro-particles (250 µL) were recovered from the suspension and washed with TRIS buffer (2 x 1000 µL, 25 mM, pH 8.0, Triton X-100 0.01 % w/v).

A TRIS buffer solution was then added (1400 µL), together with three small fragments of copper wire (~ 1 cm), a bathophenanthrolinedisulfonic acid disodium salt solution (120 µL, 6.2 mM, DMSO/water 1/1) and a **Tiiii** solution (150 uL, 12 mM, methanol/DMSO 3/1). This mixture was kept under gentle orbital stirring for 18 h. Magnetic micro-beads were then recovered and washed with TRIS buffer (3 x 1000 µL) and PBS 1X buffer (2 x 500 µL), and stored at 4°C in 500 µL of PBS 1X buffer.

Electrochemistry and Electrochemiluminescence.

The ECL measurements were carried out by depositing 50 µL of the sample directly on the screen-printed electrode (see the detection part for the details).

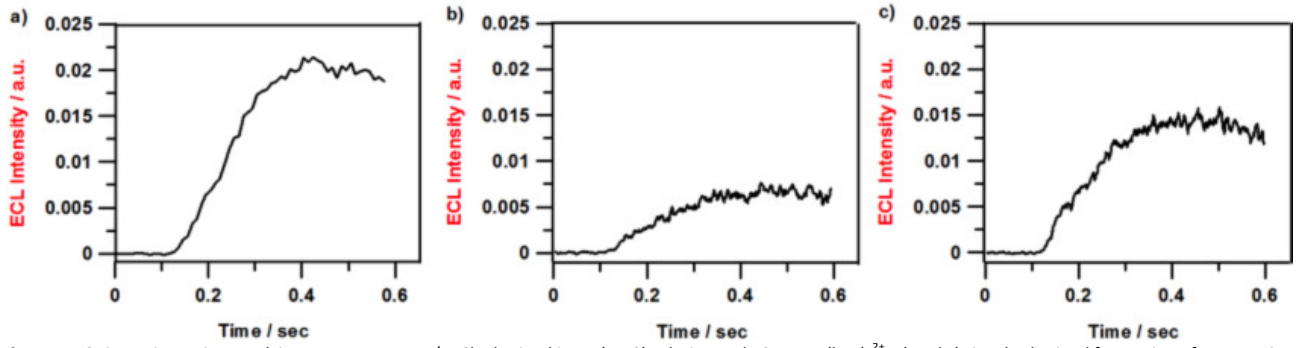
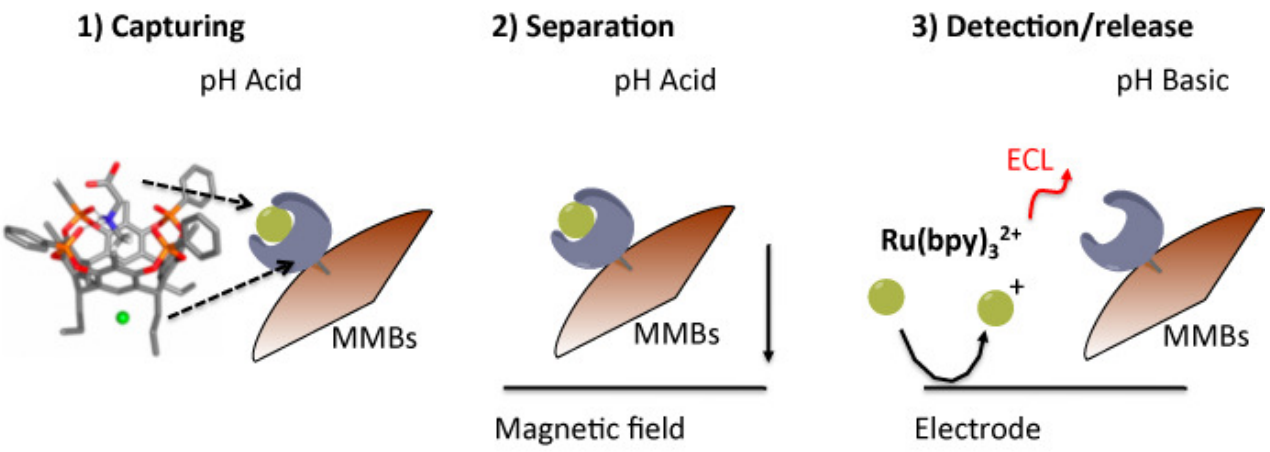


Figure 4. ECL intensity *vs* time applying 1.45 V versus Ag/AgCl, obtained in PB (pH 9) solution and 10 µM $Ru(bpy)_3^{2+}$; a) and c) signals obtained from urine of metastatic PCa samples; b) signal obtained from urine of a healthy sample. All the urine samples were centrifuged with a molecular weight cut-off membrane of 3 KDa. MMBs were previously functionalized with the **Tiiii**, after the **Tiiii@MMB** were incubate for 1 h with the urine sample adjusting the pH at 5 with PB. The **Tiiii@MMB**-Sarcosine hydrochloride complex were washed three times with 0.05% Tween 20 and PB pH 5.



Scheme 2. Schematic representation of the three steps in the sample analysis, see text. Inset, the crystal structures of complex methyl **Tiiii**-sarcosine hydrochloride.[15] C, gray; O, red; P, orange; N, blue; Cl, green; H, white; H bonds, black dotted lines. For clarity, the H atoms of the cavitand and those not involved in complexation of the guests have been omitted

The ECL signal generated by performing the potential step program was measured with a photomultiplier tube (PMT, Hamamatsu R4220p) placed, at a constant distance, on the electrode and inside a dark box.[34] A voltage in the range 550-750 V was supplied to the PMT. The light/current/voltage curves were recorded by collecting the preamplified PMT output signal (by a ultralow-noise Acton research model 181) with the second input channel of the ADC module of the AUTOLAB instrument.

Assembly of the sensor

Phosphate buffer (PB) solutions (total phosphate concentration 100 mM) at different pH (pH 5 and 12) were prepared dissolving 12 g of NaH_2PO_4 in distilled water. Triton-X surfactant was added at the solution (0.01% w/v). The pH was adjusted adding NaOH solution until the pH required was reached. The pH variation during NaOH addition was controlled by a pHmeter. Sarcosine concentration was then measured by three steps resumed in Scheme 2:

Capturing (Scheme 2a)

All samples were incubated at room temperature for one hour with PB (pH 5) under constant mixing.

Separation (Scheme 2b)

All the samples were washed using PB (pH 5) to remove the unrecognized sarcosine molecules. Before washing, magnet was applied for 5 minutes. Subsequently, the solution was removed and 200 µL of new PB (pH 5) solution were added. The magnet was removed and the samples are mixed to obtain an homogeneous suspension. This procedure was repeated twice.

Release and Detection (Scheme 2c)

Magnetic separation was applied for 5 minutes to remove the solution: 200 µL of PB (pH 12) with 20 µM of $Ru[(bpy)_3]Cl_2$ solution was then added and the beads were resuspended by vortexing. Disposable Carbon Screen Print Electrodes, SPEs (C 110 DropSens®[35]), were used as working electrodes. 50 µL of the sample were deposited on the SPE electrode and ECL was measured throughout chronoamperometry technique (0V, 1s ; 1.4V, 4s; PMT=750V).

Urine treatment

The urine samples were collected in the University Hospital of Verona according to the rules of the local ethical committee. Individual human urine samples (5 mL) were loaded onto 15 mL ultrafiltration filters with a molecular weight cut-off of 3,000 Da and centrifuged at 8000 g at 15 °C, finally adjusting the urine pH to 5.

Conclusions

We demonstrated for the first time the application of sarcosine as co-reactant for the ECL generation. The grafting of the supramolecular recognition unit **Tiiii** on magnetic micro-beads led to a cheap disposable device for sarcosine detection, a new biological marker applied to PCa diagnosis. The potential application of the sensor presented here was tested by quantifying sarcosine in complex matrix such as urine. A correlation between the concentration of well-known PCa markers such as PSA and PSMA in serum and the sarcosine concentration in the urine is now under investigation in our laboratory.

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