Selective Amperometric Detection of Sarcosine, Creatine and Creatinine at Sputtered Ru/Rh Electrode Layers

by Walter Vastarella, Dirk Janasek, Uwe Spohn

Selective detection of sarcosine, creatine and creatinine is still a topical issue in clinical applications. The possibility to detect amperometrically and selectively the hydrogen peroxide cathodic reduction at ruthenium and rhodium modified gold electrode at potential of -100 mV vs. Ag/AgCl/0.1 M KCl was early showed. In this paper sarcosine has been selectively detected in the concentration range between 5 μM e 1 mM, by using a sarcosine oxidase modified membrane onto the multilayer electrode, after an accurate selection of optimal flow rate and pH conditions. Combining the sarcosine sensor with a packed co-immobilised creatinase and creatininase enzyme reactor, the detection of creatine and creatinine has been performed respectively between the range 20-1,000 μM and 10-1,000 μM, with a relatively good operational stability under flow conditions. This represents a good basis to enhance novel biosensing systems in order to detect selectively and sensitively creatine and creatinine.

The selective and sensitive detection of sarcosine, as proof of the presence of creatine and creatinine, have attracted much attention during the last decade in the development and application of amperometric biosensors, due to its importance for clinical and biomedical purposes [1].
Nowadays, most clinical laboratories and commercial analyzers continue to use for creatine detection a spectrophotometric procedure based on Jaffé reaction, where the absorbance of the addition complex, resulting from the reaction between creatinine and picrate, is monitored in alkaline solution [2]. The reaction is not specific for creatinine, because many chromogenic compounds interfere in this assay, thus the selective creatinine evaluation remains a problem [3]. Consequently, alternative methods using high performance liquid chromatography [4] or enzyme-based methods were invented with a view to overcoming these problems [5, 6]. Sarcosine oxidase has been widely used in the enzymatic methods. Recently new systems with integrated miniaturised sensing elements were developed, even using microelectronics techniques for electrodes fabrication, but so far all these electrodes showed relatively low operational stability, rather high costs and long response time. Tsuchida and co-workers [7] designed new multi-enzymatic amperometric sensors based on the following catalytic sequence of reactions:

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$$\text{creatinine} + H_2O \xleftrightarrow{\text{CA}} \text{creatine} \quad (1)$$

$$\text{creatine} + H_2O \xleftrightarrow{\text{CI}} \text{sarcosine} + \text{urea} \quad (2)$$

$$\text{sarcosine} + H_2O + O_2 \xleftrightarrow{\text{SOD}} \text{glycine} + HCHO + H_2O_2 \quad (3)$$

where reactions were catalysed respectively by creatininase (1), creatinase (2) and sarcosine oxidase (3). When these enzymes are coupled, the current responses resulting from the catalytic reaction are diminished, thus affecting the sensitivity and detection limit of these methods. On the basis of the work of Tsuchida, new commercial electrode-based creatinine sensors were designed as clinical analysers (Nova 16). After much efforts, a disposable *biochip* sensors for the creatinine determination was developed by Maradas and co-workers [8], in which electropolymerized perm-selective films were used. In their publication the fabricated sensors were tested in a flow injection set-up; detection limits, long term operational stability and selectivity were also reported.
One of the drawbacks of these miniaturised sensors is the very low signal of creatinine for samples with high creatine content, because of the high sensitivity for creatine. The topic demand to have a creatine and creatinine sensor with both good operational stability and high specificity remains. We have seen that recently a large amounts of metal-dispersed modified electrodes was prepared in order to reduce the inter-

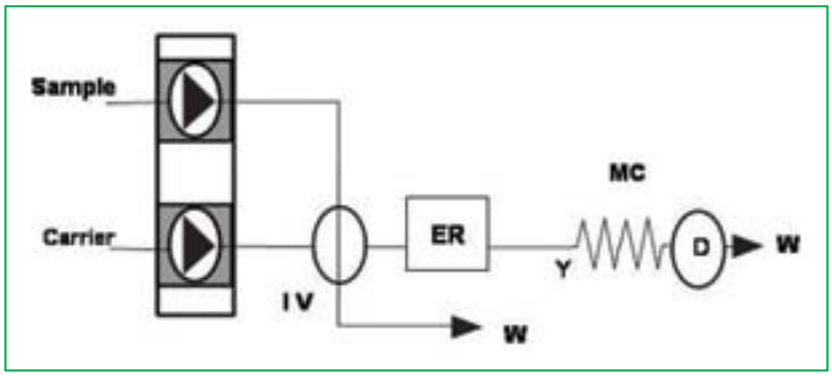

Figure 1 - FIA set-up: sample is injected by peristaltic pump; IV: injection valve; ER: enzyme reactor; MC: mixing coil; D: flow detector cell; W: waste

ferences of several electroactive substances at the detection potential of hydrogen peroxide [9]. Guilbault and coworkers used for the first time the cathodic deposition of ruthenium and rhodium layers on glassy carbon electrodes in order to achieve low working detection potentials, without giving information about the background current and the ratio of the signal to background current.
We have already shown electrochemical investigations of ruthenium and rhodium layer deposited after each other on gold electrodes, onto which a glucose oxidase modified membrane was placed.
The same FIA methodology for the enzymatic detection of sarcosine, based on amperometric investigation by means of a sarcosine oxidase modified electrode, has been used, with a suitable modification of the FIA set-up and addition of a multi-enzymatic cartridge-reactor. Sarcosine oxidase (SOD) catalyzes the reaction (3) to give glycine, formaldehyde and hydrogen peroxide as products. The cathodic reduction of H_2O_2 has been showed to be successfully detected by ruthenium/rhodium on gold electrode at a potential of - 100mV vs. Ag/AgCl/0.1 M KCl, where the influence of several electroactive non-enzymatic substances is minimized.

Materials and methods

Chemicals and reagents

L-ascorbic acid, uric acid, paracetamol, glycine, glutaraldehyde (GA) 25% acqueous solution, sarcosine, sarcosine oxidase from *Bacillus Species* (EC 1.5.3.1, cat.Nr. S-7897, with 47 and 50 units/mg solid), creatine, creatinine and bovine serum albumine (BSA, cat. nr. A-9647) were purchased from Sigma (Deisenhofen, Germany) and used without further purification. Creatinase (EC 3.5.3.3) and creatininase (EC 3.5.2.10) were from Sigma Aldrich.
Controlled porous glasses (CPGs) sphere of the sieve fraction from 140 to 160 mm were obtained from Schuller (GmbH, Steinach, Germany). All other chemicals were of analytical grade and from Merck (Darmstadt-Germany). 0.2 M sodium/potassium phosphate buffer solutions containing 0.2 M KCl were prepared from doubly-distilled water and used as carrier solution.

Measuring set-up

Figure 1 shows the FIA set-up which was used to investigate the sarcosine, creatine and creatinine detection. Sample solutions were injected into the buffer carrier solution by a pneumatically actuated injection valve ***IV*** (Rheodyne Cotati, USA) with a sample loop of 30 µl and reacted in the enzyme reactor ER.
At the mixing point ***Y*** and in the mixing coil ***MC*** the carrier was mixed with the sample and pumped in the detection cell ***D*** .The electrochemical signal was monitored and scanned using a software controlling the pump and the injection valve simultaneously. The amperometric detector ***D*** was a thin layer flow cell consisting of two Plexiglas plates, into one of which a groove was milled for lodging the indicator electrode. The effective geometric area for the indicator electrode was 20 mm^2.
The counter electrode was a stainless steel capillary mounted at the outlet of the detection cell. Every potential indicated in the following experiments was measured with respect to a Ag/AgCl/0.1 M KCl reference electrode. Hydrodynamic voltammograms and cathodic current were recorded again with the home-made potentiostat CPE-1, under flow conditions. To prepare the indicator electrodes thin and clean gold foils of thickness of 0.025 mm were modified by Ru/Rh layers, as previously described [10].
The plasma chemical deposition process as well as the microscopic measurements about layers thickness and roughness were performed at the Fraunhofer Institute of Material Science Freiburg/Halle. All the next experiments were performed onto the multilayers electrode, sputtering 100 nm of Ru after deposition of 7.5 nm thick Rh on gold.

Enzyme immobilisation and preparation of the enzymatic reactor

Sarcosine oxidase from *Bacillus Species* was immobilized covalently onto a pre-activated Nylon ID-membrane (Immunodyne, Pall, Dreieich, Germany) by using the following procedures: the content of 50 unit per mg of sarcosine oxidase (SOD) was dissolved in 400 µl of 0.9 mol l^{-1} trehalose in 0.1 M sodium/potassium phosphate buffer at pH 8.0.
Thereafter a concentration procedure was performed to obtain 120 µl and a solution of 20 µl were dropped onto the ID-membrane strip. The co-immobilised creatine and creatinine enzymatic reactor was designed compatibly with the flow

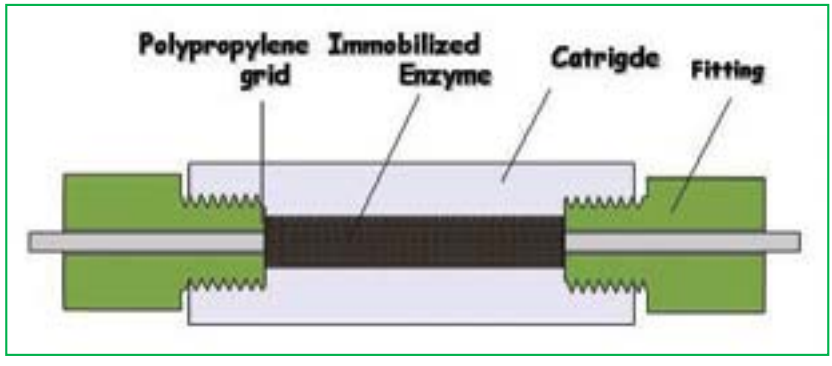

Figure 2 - Detailed scheme of a complete packed enzyme cartridge

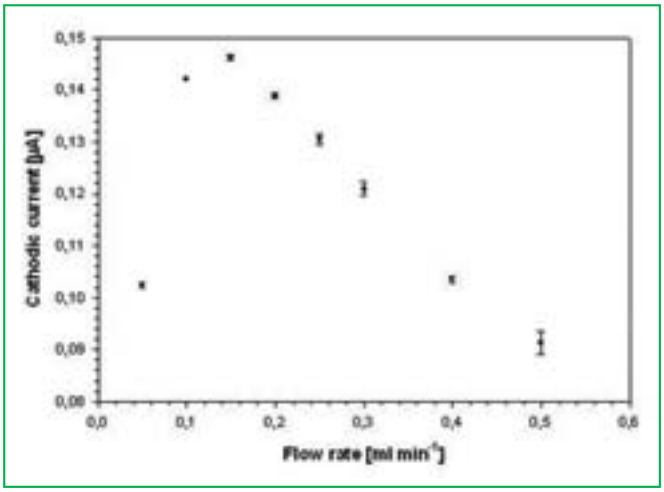

Figure 3 - Cathodic current dependence on the flow-rate. Sarcosine oxidase modified membrane was put on Ru (100 nm)-Rh (7.5 nm)-gold electrode. [Sarc]=0.5 mM in 0.1 M sodium and potassium phosphate buffer (pH 8.0); E_{el}=-100 mV; injection volume: 50 µl

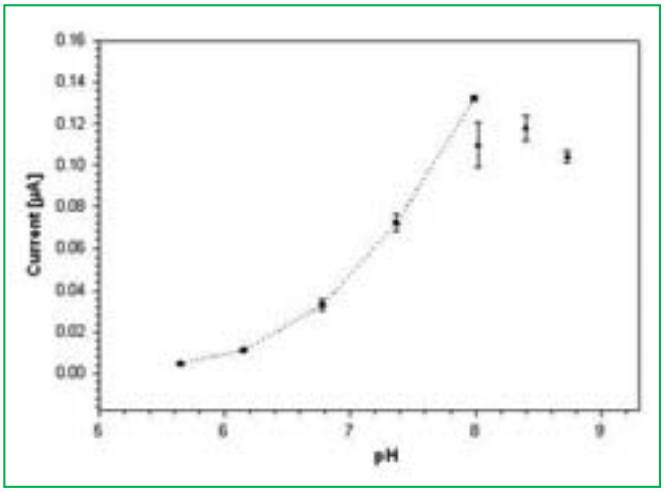

Figure 4 - Sarcosine signal dependence on the buffer pH. Sarcosine oxidase modified membrane was put on Ru (100 nm)-Rh (7.5 nm)-gold electrode; flow rate v_D: 0.15 ml min.$^{-1}$; injection volume: 50 µl, E_{el}=-100 mV, [Sarc]=0.5 mM; (•)=0.2 M sodium-potassium buffer +0.2 M KCl, (▲)=0.2 M TRIS buffer +0.2 M KCl

conditions and was packed in a cartridge according to the method proposed by Spohn and co-workers [11, 13]: 200 mg of aminosilylated Trisoperl CPGs were suspended in 5 ml of 2.5% m/m glutaric aldehyde in 0.1 M phosphate buffer at pH 6.5 and were activated by water jet in vacuum for 30 min. and under normal pressure for further 30 min. The suspension was filtered over a fritted glass with doubly distilled water under *vacuum* conditions.

A total amount of 18.4 mg of lyophilized cretininase/creatinase enzyme mixture, necessary to have respectively 189.6 unit of creatininase and 192 units of creatinase, was dissolved in phosphate buffer at pH 8.0; pre-activated CPGs was added to the enzyme solution

The obtained suspension was kept under reduced pressure for 30 min. at ambient pressure and then at a temperature of 4 °C for further 15 min. The suspension was packed into the enzyme cartridge to give an enzyme reactor of 2 cm length, which is schematically showed in Figure 2. Finally the enzyme reactor was connected by tubing, upward of the detection cell.

Table 1 - Selectivity of the amperometric sarcosine detection under FIA conditions; flow rate v_D =0.15 ml min.$^{-1}$, level of confidence α = 0.05, n=number of repeated measurements

Interferent	*Relative peak height in the presence of sarcosine %*	*Relative peak height in the absence of sarcosine %*	*Relative decrease of the glucose signal after injection of interferent %*
Without	100.0	0.0	
Ascorbic acid (10 µM)	82.1±0.2	-41.65±0.13	0.0±0.2
	n=6	n=5	n=5
Glycine (1 µM)	99.89±0.10	0.0±0.0	0.00±0.13
	n=6		n=4
Formaldehyde (1 µM)	90.51±0.11	0.0±0.0	1.5±0.2
	n=9		n=8
Paracetamol (1 µM)	100.41±0.06	0.0±0.0	0.58±0.07
	n=5		n=5

Result and discussion

Adjustment of the experimental parameters

Figure 3 shows the dependency of the cathodic current on the detector flow rate, as the sarcosine concentration and the applied potential were kept constant respectively to 0.5 mM and –100 mV. The signal current is increasing with increasing the flow rate in the range between 0.05 and 0.15 ml min.$^{-1}$, whereas decrease in the range from 0.15 and 0.50 ml min.$^{-1}$. Taking into account that the maximum is reached at 0.15 ml min.$^{-1}$ the following experiments were performed at this flow rate value.

In Figure 4 is reported the buffer pH dependence of sarcosine detection on the current signal; although the sensitivity is relatively high in a wide pH range, the highest signal was reached at pH 8.0, by using a 0.2 M sodium/potassium phosphate buffer containing 0.2 M KCl, to confirm the right buffer conditions we have always used. The selectivity of the proposed indicator electrode in the amperometric biosensor is restricted by the anodic oxidation and sometimes also by the cathodic reduction of interferents, which can cause electrode fouling and poisoning.

Hence the influence of electrochemically active compounds was investigated both in presence and absence of sarcosine.

In order to evaluate the selectivity of the electrode, 0.25 mM of sarcosine solution was mixed continuously with 0.5 mM of interferent solutions and/or phosphate carrier buffer, under flow conditions, at a flow rate ratio of 1:1 just before the injection valve. Table 1 summarizes the results. The relative peak heights measured both in

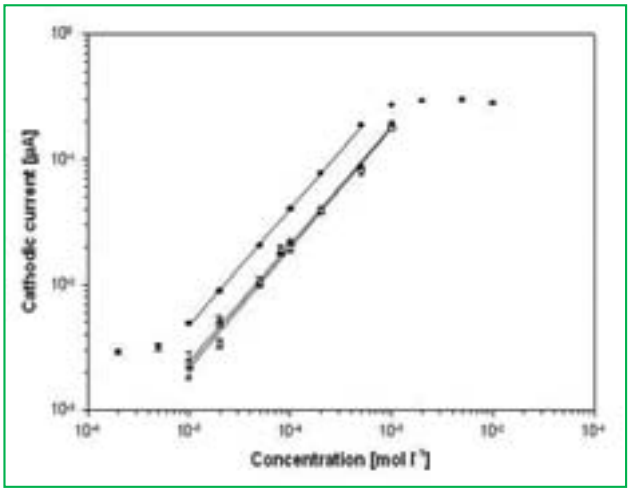

Table 2 - Sensor parameters for sarcosine,creatine and creatinine calibrations. Regression equation: lg (h/mV)=a lg (c/mol l^{-1})+b

	Detection limit [mol lt-1]	*Detection range [mol lt-1]*	*a*	*b*	*R2 (α=0.05), [%]*
Sarcosine	2 10^{-6}	1 10^{-5}÷5 10^{-4}	0.93	2.34	99.97
					m=7;
					n=4
Creatine	1 10^{-5}	10^{-5}÷10^{-3}	0.94	2.07	99.74
					m=8;
					n=3
Creatinine	1 10^{-5}	10^{-5}÷10^{-3}	0.95	2.10	99.34
					m=8;
					n=3

Figure 5 - Calibration graph of the amperometric sarcosine, creatine and creatinine detection flow rate v_D: 0.15 ml min.$^{-1}$, E_{el}=-100 mV; (▲)=sarcosine, (❑)=creatine, (•)=creatinine

absence and in presence of sarcosine were related to the peak height measured for 0.25 mM sarcosine, in absence of any interferent. Only ascorbic acid showed anodic interference, because of its probable re-oxidation process. Glycine showed neither electrode poisoning effect neither significant anodic interference.

Sarcosine, creatine and creatinine detection

Figure 5 shows the double logarithmic calibration graphs of sarcosine, creatine and creatinine, supporting by Table 2 where the main calibration parameters are summarized. The detection of sarcosine was performed after placing onto the indicator electrode the SOD modified membrane, which was prepared according to the method described in the previous section. The calibration curve showed a linear dynamic range between 10 μM and 500 μM.
The creatine and creatinine detection was measured after the enzyme cartridge preparation and recorded in the range between 10 μM and 1 mM. All the measurements were performed under flow conditions with an injection volume of 50 μl. The double logarithmic regression functions can be described by the following:

$$\lg (\boldsymbol{h}/\text{mV}) = (0.933\pm0.016)\ \lg (\boldsymbol{c}/\text{M}) + (2.34\pm0.04) \quad (4)$$

$$\lg (\boldsymbol{h}/\text{mV}) = (0.938\pm0.011)\ \lg (\boldsymbol{c}/\text{M}) + (2.07\pm0.04) \quad (5)$$

$$\lg (\boldsymbol{h}/\text{mV}) = (0.95\pm0.03)\ \lg (\boldsymbol{c}/\text{M}) + (2.10\pm0.04) \quad (6)$$

where ***h*** is the peak height and ***c*** the analyte concentration.The detection limits were evaluated at the dispersion factor of $\mathbf{D_{max}}$=3.3, which was determined according to Rûzicka and Hansen relationship [14].
The high overlapping between creatine and creatinine calibration curves showed that the hydrolase enzyme should work quantitatively in reaction (1). Conversely, the difference between creatine/creatinine curves compared to the sarcosine calibration demonstrated that reaction (2) do not work quantitatively. In every case enzyme activity should not to decrease under flow conditions in the enzymatic reactor. The SOD dependent membrane onto the indicator electrode showed a high long term operational stability, which was determined by the sequential injection of 0.5 mM sarcosine. After a period of around 5 hours (100 injections), during which the electrode surface was forming and the signal was not stabilized, a relatively stable peak signal height was achieved for the next 19 hours, thus confirming the relatively good capability of the immunodyne membranes to keep the quality of the enzyme properties.
The Figure 6 shows the signal reduction in 24 hours of sequential injections for the protected sarcosine oxidase sensor. The peak width was only about 40 s, depending on the injected sarcosine concentration, allowing a frequency of 20 injections per hour. When using smaller flow cell and sampling volume or doubling the flow rate, injection frequency would be enhanced, enabling fast calibration during on-line analysis, but at the same time, the signal sensitivity would undergo a strong decrease. The lifetime of the sensor seemed to be also relatively good. Considering that the novel system should be subjected to further improvements, at the present state, it could be used for more than 400 determinations over a period of two-three week.

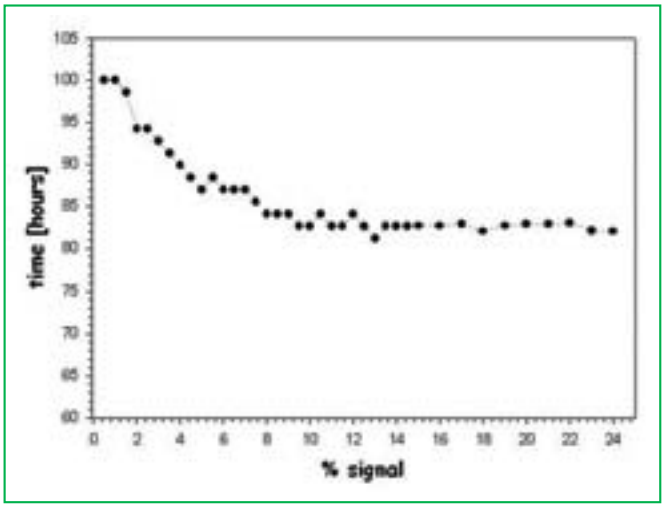

Figure 6 - Long term stability of the immunodyne protected sarcosine-sensor. 100 sequential injections of 0.5 mM sarcosine have been showed

Conclusions

After deposition of 100 nm Ru followed by approximately 7.5 nm Rh layers, modified gold electrodes can be used to detect sarcosine amperometrically at potential of –100 mV vs. Ag/AgCl/0.1 M KCl. Both a relatively high operational stability and a high selectivity against many electroactive substances were achieved.
The proposed electrode was used in combination with a multi-enzymatic creatininase/creatinase flow-cartridge, to detect not only sarcosine, but also creatine and creatinine, in biosensing systems where indicator electrode is covered by sarcosine oxidase modified membrane layers.
The sensor activity is relatively high in a broad range of pH and flow rate. The optimum pH of 8.0 is favourable for a number of possible applications. All the experiments described in this chapter show that a stable first generation biosensor based on sarcosine oxidase immobilized on highly porous membranes and creatinase-creatininase multienzymatic cartridge can be constructed. The present biosensor can compete with other first generation biosensor for sarcosine detection described in literature.

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