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Direct determination of copper in urine using a sol–gel optical sensor coupled to a multicommutated flow system

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Abstract In this work, a multicommutated flow system incorporating a sol–gel optical sensor is proposed for direct spectrophotometric determination of Cu(II) in urine. The optical sensor was developed by physical entrapment of 4-(2-pyridylazo)resorcinol (PAR) in sol–gel thin films by means of a base-catalysed process. The immobilised PAR formed a red 2:1 complex with Cu(II) with maximum absorbance at 500 nm. Optical transduction was based on a dual-colour light-emitting diode (LED) (green/red) light source and a photodiode detector. The sensor had optimum response and good selectivity towards Cu(II) at pH 7.0 and its regeneration was accomplished with picolinic acid. Linear response was obtained for Cu(II) concentrations between 5.0 and 80.0 $\mu\text{g L}^{-1}$, with a detection limit of 3.0 $\mu\text{g L}^{-1}$ and sampling frequency of 14 samples h^{-1} . Interference from foreign ions was studied at a 10:1 (w/w) ion:Cu(II) ratio. Results obtained from analysis of urine samples were in very good agreement with those obtained by inductively coupled plasma mass spectrometry (ICP–MS); there was no significant differences at a confidence level of 95%.

Keywords Cu(II) optical sensor · Sol–gel · 4-(2-Pyridylazo)resorcinol · Multicommutation · Urinary copper

Introduction

Copper is an essential trace element for the catalytic activity of many enzymes involved in biological processes. It is required for haemoglobin synthesis and is a constituent of several metalloenzymes with oxidase activity. Under normal conditions the urinary excretion of copper is very low, usually less than 40 $\mu\text{g day}^{-1}$ [1, 2]. However, this value is increased in several pathologies related to abnormalities in copper metabolism. The most important is Wilson's disease, or hepatolenticular degeneration, that results in excessive accumulation of copper in the liver, brain, cornea and kidneys. There is also an increased urinary output of copper, up to 500–1,000 $\mu\text{g day}^{-1}$ [2]. Hence, the determination of copper in urine is of particular interest in clinical chemistry for purposes of diagnosis, for monitoring Wilson's patients under chelation therapy, for detection of environmental or occupational exposure, and for nutritional studies.

Determination of copper in urine is usually performed by relatively expensive techniques, such as graphite furnace atomic absorption spectrometry (GFAAS) [3–6], atomic absorption spectrometry (AAS) [7–9] and inductively coupled plasma mass spectrometry (ICP–MS) [10–12]. There are some colorimetric methods for measurement of urine copper levels [13–15], but they are time-consuming and usually require previous ashing and/or chelation followed by extraction.

Several optodes have been described for sensing of Cu(II) ions; these are based on indicators such as zincon [16], PAN [17, 18], 1,2-cyclohexanedione thiosemicarbazone [19], DPAN [20], lucifer yellow [21], pyrocathocol violet [22], and cupron [23]. All suffer from long response times and high detection limits. Some have been applied to the analysis of waters or alloys [17–20], but most lack analytical application.

PAR (4-(2-pyridylazo)resorcinol) is a non-selective azo dye widely used as a colorimetric reagent for metal ions because it forms very stable water-soluble chelates with most transition metals. At pH values above 5.0,

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PAR forms a 2:1 complex with Cu(II) with molar absorptivity of $5.89 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ [24, 25]. PAR, immobilised in polymeric materials such as Dowex [26, 27], poly(ethylenimine) [28], and XAD-4 and XAD-7 [27], has been used for the determination of Cu(II) ions.

Sol-gel glass [29, 30] is a good host matrix for development of viable chemical sensors, as it provides a physically and chemically stable environment with excellent optical transparency and can be cast in different shapes and sizes, including fibres, monoliths and thin films [31, 32]. By use of the sol-gel process inorganic networks can be obtained in a simple and versatile way, by hydrolysis and condensation of silicon alkoxides at room temperature. Sensing agents can be readily entrapped in the porous glass matrix during the steps of the process, preserving their selectivity and activity.

In this work, PAR was immobilised in sol-gel thin films to obtain a Cu(II) optical sensor. The proposed sensor was then coupled to a continuous flow system to accomplish the direct determination of urinary copper, in order to achieve simpler, cleaner, and biohazard-protected handling of samples.

Experimental

Reagents and solutions

All reagents were of analytical grade and used without further purification, unless stated otherwise. Milli-Q (Millipore) deionised water, with resistivity above $18 \text{ M}\Omega \text{ cm}^{-1}$, was used to prepare all solutions.

Tetraethoxysilane (TEOS; product no. 86578), 4-(2-pyridylazo)resorcinol (PAR; product no. 82970), and nitric acid (product no. 84385) were purchased from Fluka. 3-Aminopropyltriethoxysilane (APTES, product no. A-3648) and picolinic acid (product no. P-5503) were purchased from Sigma. Tetramethylammonium hydroxide, 25 wt% solution in water, (TMAOH; Aldrich, product no. 33,163-5) and ethanol (Merck, product no. 1.00983) were also used. Working Cu(II) solutions were obtained by adequate dilution of a $1,000\text{-mg L}^{-1}$ SpectrosoL (BDH, product no. 141394P) standard stock solution. Buffer solutions with pH values ranging from 3.0 to 10.0 were prepared as recommended [33] with analytical grade reagents from Merck.

Instrumentation

Visible spectra for characterization of the sensors were acquired with a Hewlett-Packard (Palo Alto, CA, USA) model 8452A diode-array spectrophotometer. Surface area, total volume of pores, and average pore volume were measured with a Micromeritics ASAP 2000 apparatus (Mönchengladbach, Germany) by N_2 adsorption/desorption analysis. The homogeneity of the sensing membranes and their microstructure were evaluated by scanning electron microscopy (SEM)

using a Jeol (Peabody, MA, USA) JSM-6360 microscope and atomic force microscopy (AFM) with a TopoMetrix (Santa Clara, CA, USA) Discover TMX2010 AF microscope.

The sensing membranes were placed in a flow-cell (Fig. 1) constructed specifically for this purpose with two perspex blocks and a silicone rubber spacer. The flow manifold used for the determination of Cu(II) is shown in Fig. 2. It was built with three NResearch (Stow, A, USA) 161 T031 three-way solenoid valves and a Gilson (Villiers-le-Bel, France) Minipuls 3 peristaltic pump equipped with isoversinic pumping tube of the same brand. Flow lines and mixing coil were made of 0.8 mm i.d. PTFE tubing. Homemade connectors and confluence points were also used. For optical transduction two light emitting diodes (LED), green (500 nm) and red (650 nm), from RS Components (Northants, UK), were employed as light sources connected to plastic optical fibres (ref. FOP1-ST), a silicon photodiode detector (ref. VISD), and a photodetector amplifier (ref. PDA1) from WPI (World Precision Instruments, FL, USA). Control of the photometer and commutation devices and data acquisition, were accomplished with VisiDAQ 3.1 Standard software, using a microcomputer equipped with a PCL-818 L Advantech interface card.

Inductively coupled plasma mass spectrometry determinations were made with a PlasmaQuad 3 ICP mass spectrometer from VG Elemental (Cheshire, UK).

Preparation of the sensor

The Cu(II) sensor was obtained by entrapment of 4-(2-pyridylazo)resorcinol (PAR) in thin films prepared by

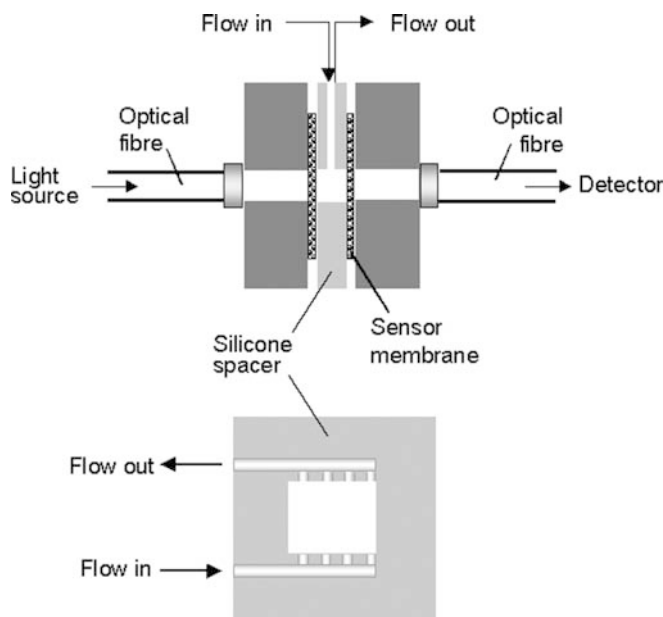
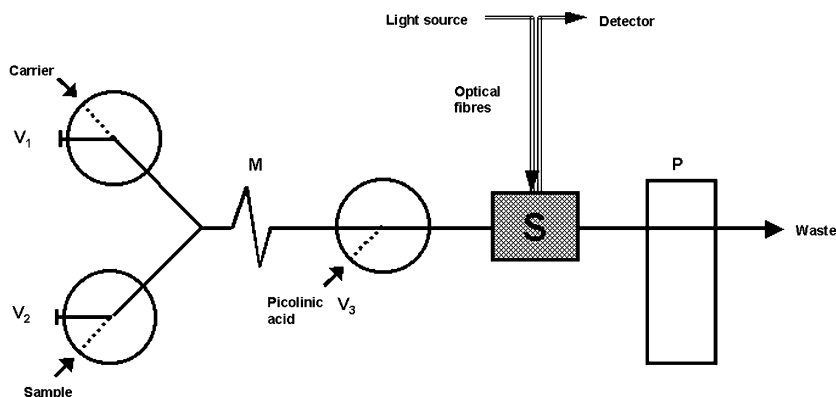


Fig. 1 Schematic diagram of the flow cell, cross-sectional view, and the silicone spacer (3 mm thick), front view

Fig. 2 Diagram of the flow manifold. V_1 , V_2 and V_3 are three-way solenoid valves; the dotted line corresponds to the activated position and the continuous line to the “off” position. M mixing coil, 25 cm long; S sensor membrane; P peristaltic pump



the sol-gel method. Glass slides (18×18 mm) were used as substrates for film deposition. Prior to coating the substrates were treated with concentrated nitric acid and ethanol, thoroughly rinsed with deionised water, and dried at 100 °C, in order to activate the silanol groups on the surface of the glass.

To prepare the sol solution 3.0 mL TEOS, 1.0 mL APTES, 16.0 mL ethanolic PAR solution, and 2.2 mL TMAOH were mixed and mechanically stirred in a PTFE beaker for 4 h at room temperature. The molar ratio Si:H₂O:ethanol was 1:4:12 and the final PAR concentration in the sol was 1.0 g L⁻¹. Thin films were obtained by spin-coating 100.0 μL sol on the glass substrates at 3,000 rpm for 15 s, which were then allowed to gel and age at room temperature for at least 5 days before use.

Procedures

The optical response of the sensor was studied by means of absorption spectra recorded in the wavelength range 400–700 nm after its immersion in 4.0 mg L⁻¹ copper solutions at different pH, from 3.0 to 10.0. Surface area and porosity of the membranes were estimated by recording the N₂ adsorption/desorption isotherms at 77 K on the full range of relative pressure P/P_0 . Total volume of pores and surface area were calculated directly, based on the mass of adsorbent after adsorption vs. pressure. Average pore size was calculated as $4 \times \text{volume} / \text{surface area}$ from the BET equation. SEM and AFM were used to evaluate morphology and microstructure of the porous thin films. The extent of leaching was evaluated by recording the absorbance of the sensor at the wavelength 410 nm, immersed in pH 7.0 K₂HPO₄/NaOH buffer solution (selected carrier solution), in 2-min intervals.

Two sensing layers were placed in a flow cell that was constructed in-house, as depicted in Fig. 1, and coupled to a flow set-up assembled according to Fig. 2. Three-way solenoid valves V_1 , V_2 , and V_3 were responsible for access of carrier, sample and regenerating solutions, respectively, according to a programmed activation cycle. First, valves V_1 and V_2 were

simultaneously activated for 25 cycles, ON/OFF and OFF/OFF, respectively, lasting 4 s each, allowing the alternate insertion of small plugs of sample and carrier solution, which after mixing in a 25-cm long coil (M), flowed through the flow cell containing the sensor. By selecting different ON/OFF time ratios, solutions of different concentrations were produced, and so calibrations were performed automatically using the Cu(II) standard stock solution. When the complexation reaction was complete, regenerating solution was inserted in the flow cell by continuous activation of valve V_3 for 100 s. After establishment of the baseline (corresponding to the absorbance of the immobilised PAR alone), valve V_1 was activated for 50 s, inserting carrier solution into the system for washing purposes. Flow rate was fixed at 0.5 mL min⁻¹. The concentration of the pH 7.0 buffer solution was optimised in order to obtain maximum sensitivity. NH₃, EDTA, thiocyanate, citrate, tartrate, oxalate, thiourea, HCl, and picolinic acid, at concentrations between 0.01 and 1.0 mol L⁻¹, were investigated as potential regeneration agents for the sensors.

Absorbance of the complex was monitored using a combination of two LEDs (green/red) and a photodiode detector, resorting to a dual wavelength strategy. Successive measurements were performed at the absorption maximum (500 nm, green LED) and at a reference wavelength (650 nm, red LED). The difference between these two absorbencies constituted the analytical signal.

Results and discussion

Characteristics of the sensor

In the sol-gel process optically transparent silicate gels are synthesised by hydrolysis and condensation of monomeric alkoxide precursors, employing a mineral acid or a base as catalyst [29, 30].

In this work TEOS was co-polymerised with 3-aminopropyltriethoxysilane (APTES) by a base-catalysed process. APTES is one of the most frequent organosilane agents used for development of sensors. It enables efficient bonding between organic indicators and

inorganic substrates, improving the uptake of the immobilised dye into the sol-gel network [34]. It also results in more stable structures and eases diffusion of the analytes into the films [35].

Whereas use of acid catalysts lead to protonation of the amino-group and immediate precipitation of APTES, resulting in muddy double-phased gels, not suitable for optical applications, the basic catalyst TMAOH enables relatively large amounts of APTES to be completely incorporated in the gel network and reduces the long gelation times usually associated with this organosilane, rendering clear single-phased homogeneous gels [36]. Basic medium also has a positive effect on the complexation mechanism of PAR, preventing ionisation of the *p*-hydroxyl group in the PAR molecule, which occurs in acidic medium [37].

The water:alkoxide ratio (R) has a very important effect on the porosity characteristics of sol-gel thin films. An increase in R leads to a decrease in thickness, shrinkage, and pore volume [29, 38]. Thus, R was adjusted to 4 to ensure formation of thin films and to balance the high porosity typical of base-catalysed structures, minimising leaching problems.

The coloured coatings obtained by the previously described procedure had good optical transparency and were crack-free. Morphologies observed by SEM (Fig. 3) and AFM techniques show a network of spherical particles packed together, suggesting that the polymerisation process starts with formation of colloidal silica globules followed by their aggregation. This is in agreement with a literature report that base-catalysed hydrolysis tends to produce highly condensed particulate sols [29]. The values of $1.3299 \text{ m}^2 \text{ g}^{-1}$ for surface area, $0.02224 \text{ cm}^3 \text{ g}^{-1}$ for total volume of pores, and 51.21 nm for the average pore diameter, obtained by

nitrogen sorption porosimetry, indicate mesoporosity of the films.

Absorbance spectra recorded for the films (Fig. 4) show that the formation of a complex between PAR and Cu(II) causes a decrease of absorbance at 410 nm (maximum for PAR), and the appearance of a new peak at 500 nm. It can also be seen that the optimum pH for complex formation is 7.0, for which higher response towards Cu(II) is obtained. The films exhibited show leaching when immersed in pH 7.0 buffer solution (Fig. 5), as expected from the porosity results.

Flow system procedures

In the proposed system (Fig. 2), sample and carrier were added by a binary sampling process, implemented by time-based selection of sample and carrier aliquots. This strategy reduced the manipulation of solutions, minimising errors, because calibrations were made using a single Cu(II) standard solution that was diluted on-line with buffer solution by alternate activation of valves V1 and V2. The flow-rate was set at 0.5 mL min^{-1} in order to obtain the highest response with low leaching, maximizing the lifetime of the sensor. Under these conditions, 25 on/off cycles of valves V1 and V2, lasting 4 s each, were necessary to obtain maximum signal. For regeneration of the sensor, increasing activation times of valve V3, responsible for insertion of regenerating solution, were tested. Complete regeneration and stable baseline were attained after a 100-s period of activation. The washing step after regeneration was found necessary to remove all remaining regeneration solution and re-establish the optimum pH for complex formation. Without this step, the response time of the sensor be-

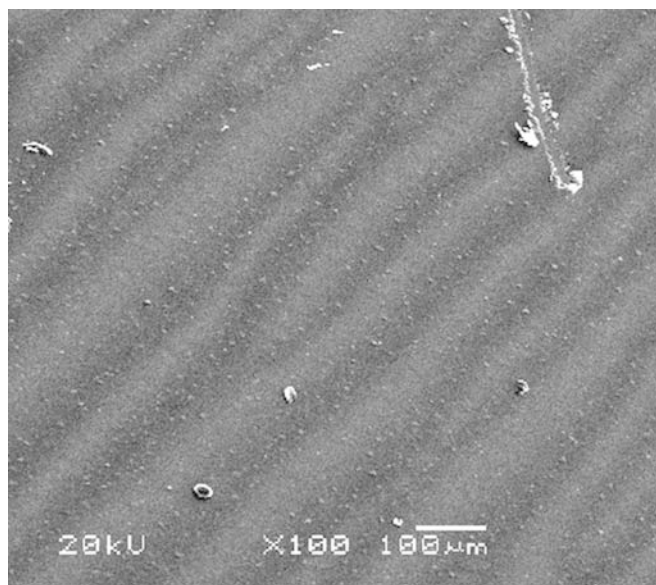


Fig. 3 Scanning electron microscopy photograph of the sol-gel thin film

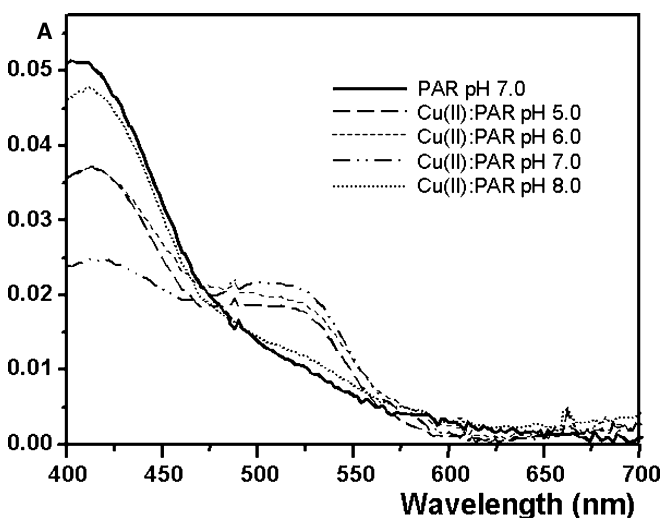


Fig. 4 Absorbance spectra of immobilised PAR at pH 7.0, and of the complex between immobilised PAR and Cu(II) at pH 5.0, 6.0, 7.0, and 8.0

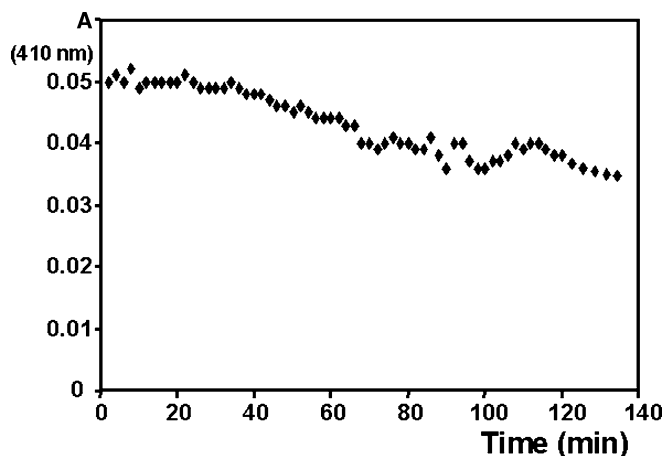


Fig. 5 Leaching profile of the sensor. Absorbance of the film immersed in pH 7.0 solution was measured at 410 nm at 2-min intervals

came very long (more than 3 min) due to the formation of pH gradients.

In order to be successfully applied in a flow system, the sensor should be rapidly and fully reversible. Several Cu(II) complexing reagents were investigated for regeneration of the sensor. Incomplete and slow regeneration, with increasing baseline drift, was observed for HCl and thiourea, and EDTA resulted in severely increased leaching, impairing the analytical signal. Other Cu(II) complexing agents, namely NH_3 , thiocyanate, citrate, tartrate and oxalate, were ineffective in regenerating the sensor; this can be attributed to the high stability of the Cu(II)–PAR complex. Picolinic acid enabled complete and rapid regeneration of the sensor and so was tested in increasing amounts from 0.01 to 0.5 mol L^{-1} . A concentration of 0.1 mol L^{-1} picolinic acid was sufficient to produce a stable baseline without damaging the sensor's useful lifetime or the response time, and so was selected as regenerating solution.

$\text{K}_2\text{HPO}_4/\text{NaOH}$ buffer solution of pH 7.0 was selected as carrier. Because urine samples are currently acidified after collection, buffer capacity became very important in this determination. To ensure that samples reached the sensor at the optimum pH for complex formation, increasing concentrations of buffer solution, from 0.1 to 0.75 mol L^{-1} , were tested. A concentration of 0.5 mol L^{-1} was found optimum to ensure maximum sensitivity, and was adopted for Cu(II) determination in the flow system.

The absorbance of the PAR–Cu(II) complex was monitored at its maximum absorbance wavelength (500 nm) and at a reference wavelength, 650 nm, at which absorbance by the PAR–Cu(II) complex and by the immobilised PAR alone were negligible (Fig. 4). However, since similar Schlieren noise [39] was observed at both wavelengths, the difference between both absorbencies minimised this physical interference.

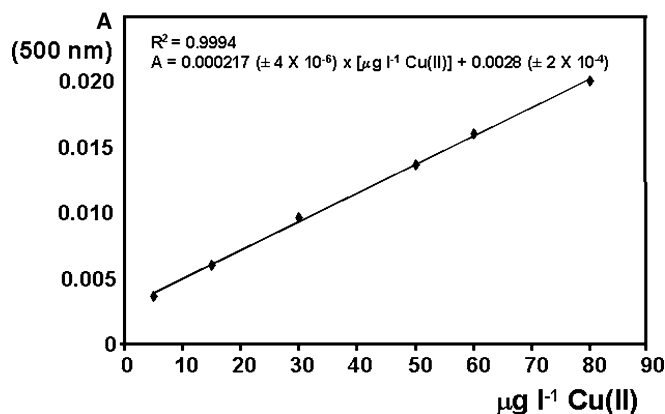


Fig. 6 Range of Cu(II) concentrations which produced a linear response, with five successive insertions of six calibration solutions

Analytical features of the method

Under the working conditions described, linear response was obtained for Cu(II) concentrations between 5.0 and 80.0 $\mu\text{g L}^{-1}$ (Fig. 6). For ten replicate measurements of a 50- $\mu\text{g L}^{-1}$ Cu(II) solution, the relative standard deviation was ca. 2%. The detection limit was estimated to be 3.0 $\mu\text{g L}^{-1}$, calculated from the standard deviation of the signals obtained by injection of a blank solution (3σ).

The useful lifetime of the sensor was evaluated by successive injection of a 50- $\mu\text{g L}^{-1}$ Cu(II) solution, and the results obtained (Fig. 7) illustrate its good stability. After 100 determinations the recorded signal showed a reduction of ca. 10%. The sensing films, stored at room temperature, were also examined over a period of several months, showing no degradation in analytical signal, response time, or sensitivity 1 year after preparation.

The proposed method can handle 14 samples h^{-1} and selectivity towards Cu(II) is good. Interference studies were carried out for determination of 50 $\mu\text{g L}^{-1}$ Cu(II) using a 10:1 (w/w) foreign ion:Cu(II) ratio. The results obtained are summarised in Table 1. The interference

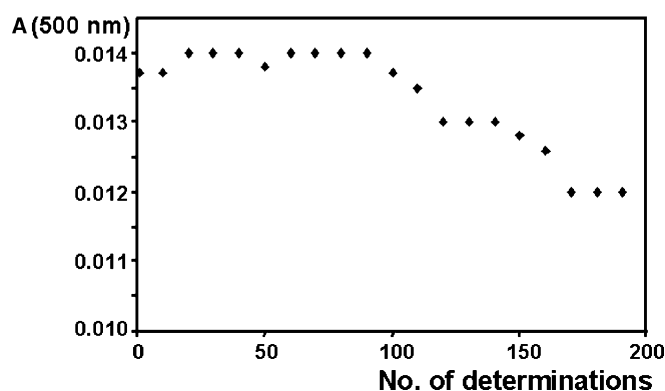


Fig. 7 Evaluation of sensor lifetime under flow conditions by successive injection of a 50 $\mu\text{g L}^{-1}$ Cu(II) solution

Table 1 Effect of some potential interfering ions on the determination of $50 \mu\text{g L}^{-1}$ Cu(II). Foreign ions were added in a 10:1 (w/w) ion:Cu(II) ratio

Ion	Change in absorbance (%)
Ni(II)	^a
Cd(II)	^a
Mn(II)	-5
Co(II)	+5
Zn(II)	^a
Ca(II)	-5
Mg(II)	^a
Na(I)	^a
K(I)	+5
Fe(II)	+11.7
Fe(III)	+16.7
Pb(II)	+21.7
Cl ⁻	^a

^aNo variation was observed

from Fe(II) and Fe(III) is due to precipitation of their hydroxides at pH 7.0, and can be eliminated by washing the system with carrier solution before regeneration. In the application of the developed sensor to urine samples Mn(II) and Co(II) interferences are not usually observed, because they occur only at concentrations much higher than physiological levels [2]. The interference levels observed for Ca(II) and K(I) remained constant, even when their concentration was higher. Because they have opposite effects their total interference does not compromise the accuracy of the measurements. Among the ions investigated, Pb(II) caused the highest interference. However, because urinary levels of Pb(II) are usually below $50 \mu\text{g L}^{-1}$ [2], the expected interference is not significant.

Analytical application

The proposed system was applied to the determination of Cu(II) in urine. The urine samples were collected in acid washed polyethylene containers, acidified (0.4% v/v nitric acid), and kept in the refrigerator until analysis. For comparison purposes, samples were spiked with different amounts of cupric nitrate. To assess the accuracy of the procedure a lyophilised control sample of urine from Seronorm (Billingstad, Norway), reconstituted according to the manufacturer's instructions with Milli-Q water, was also analysed.

The results obtained are summarised in Table 2 and are in good agreement with the values provided by ICP-MS—there is no statistical differences at a significance level of $p=0.05$. Additionally, results obtained from analysis of the reference sample match the values obtained by the laboratory responsible for certification of the sample. Data relevant to evaluation of the accuracy are summarised in Table 3 and show that the proposed method can be regarded as accurate for urinary copper determination.

Table 2 Results ($\mu\text{g L}^{-1}$) obtained from determination of Cu(II) in urine samples

Sample	Proposed method ^a	ICP-MS ^b	Relative error (%)
1	18.2 ± 1.8	17.6 ± 0.7	+3.4
2	13.4 ± 1.2	14.4 ± 0.5	-7.4
3	19.1 ± 1.7	19.1 ± 1.0	0.0
4	45.8 ± 1.5	45.1 ± 0.9	+1.3
5	42.1 ± 1.5	45.2 ± 0.1	-7.3
6	20.1 ± 1.7	20.5 ± 0.4	-2.0
7	55.0 ± 1.4	54.8 ± 0.1	+0.4
8	32.8 ± 2.3	32.7 ± 0.1	+0.3
9	44.8 ± 2.1	44.8 ± 0.9	0.0
10	46.5 ± 2.1	46.7 ± 0.1	-0.4
11	27.7 ± 2.4	28.2 ± 0.2	-1.8
12	62.0 ± 2.0	60.5 ± 2.7	+2.5
13	29.4 ± 2.3	29.2 ± 1.2	+0.7
14	24.4 ± 2.6	24.2 ± 0.1	+0.8
15	32.8 ± 2.3	33.4 ± 0.2	-1.8
16	26.8 ± 2.4	26.9 ± 1.1	-0.4
17	38.0 ± 2.2	38.0 ± 0.1	0.0
18	43.1 ± 2.2	43.7 ± 0.8	-1.4
19	39.7 ± 2.2	41.1 ± 0.6	-3.5
20	41.4 ± 2.2	43.8 ± 0.5	-5.8
21	65.4 ± 2.0	65.8 ± 1.2	-0.6
22	50.0 ± 2.1	50.1 ± 0.9	-0.2
23	62.0 ± 2.0	62.5 ± 1.9	-0.8
24 ^c	15.5 ± 1.8	15.4 ± 0.4	+0.6

^aMean from five determinations^bMean from two determinations^cReference sample**Table 3** Evaluation of the accuracy of the proposed method for determination of urinary copper

Control sample	Certified Cu(II) value ($\mu\text{g L}^{-1}$)	Obtained Cu(II) value ($\mu\text{g L}^{-1}$)	Relative error (%)
Seronorm trace elements urine	16.1 ± 1.4	15.5 ± 1.8	-3.9
Determination of copper in urine by the proposed method vs. ICP-MS			
Linear regression	Proposed method = $1.00 (\pm 0.01) \times [\text{ICP-MS}] + 0.35 (\pm 0.51)$ $R^2 = 0.9980$		
Student's <i>t</i> -test (95%)	t calculated = -1.6365; t critical = 2.068		

Conclusions

The procedure proposed in this work is a simple, sensitive, and low-cost means of determination of Cu(II) in a complex matrix, for example urine. Analysis was performed without the need for complicated sample pre-treatment procedures. The immobilisation of PAR in sol-gel thin films by means of a base-catalysed method provided a Cu(II) optical sensor with high sensitivity, selectivity, and stability, and low leaching and short response time. The flow system incorporating the optical

sensor is particularly simple and versatile and enables automatic dilution of samples, which makes this procedure convenient for the determination of higher levels of urinary copper and thus suitable for diagnosis purposes.

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