Creatinine biosensors: principles and designs

Anthony J. Killard and Malcolm R. Smyth

Creatinine biosensors, based on both potentiometric and amperometric devices, have been created. However, there are significant problems still to be addressed, including the balance between sensitivity and selectivity, interference rejection and sensor stability. In addition, many devices still rely on a dual-sensor approach for creatine and creatinine subtractive measurements. However, creatinine biosensors appear close to attaining the performance goals necessary for their widespread application. This article looks at the operating principle and design of both potentiometric and amperometric creatinine biosensors, and shows how the design of these devices affects their performance.

Creatinine has become an important clinical analyte that is used for the determination of renal and muscular dysfunction. According to Bakker *et al.*[1], it is now the most requested analyte in the clinical laboratory. Creatine is a byproduct of amino acid metabolism and is the energy source for muscle tissue. It is carried in the blood in its dehydrogenated form, creatinine. The normal clinical range for blood creatinine is 44–106 μM (Ref. 2); however, it can exceed 1000 μM in certain pathological conditions[3]. Levels >140 μM require further clinical investigation and those >530 μM indicate severe renal impairment[4].

Presently, creatinine is most widely analysed colourimetrically, using the Jaffé reaction (a procedure in which the active methylene group of creatinine reacts with alkaline sodium picrate, yielding a red–yellow complex[5]), or enzymatically[6–8]. However, neither method is convenient and both are prone to interference; for example, from the presence of ammonium ions (NH_4^+) in blood and urine.

Electrodes and, more recently, biosensor systems have many advantages over other techniques used to analyse creatinine in the clinical laboratory. They reduce the time, complexity and cost of routine clinical analyses in many areas – in particular, for analyses of blood gas, electrolytes and glucose[9]. This has been the impetus for the development of biosensor-based methods of analysis for many clinical analytes, including creatinine.

The development of creatinine biosensors has followed two paths; systems are based on either potentiometric or amperometric detection. Each system is characterized by certain advantages and disadvantages. Trends in the literature indicate that the amperometric option is becoming more popular and the first system to be successfully commercialized was an amperometric device (Nova Biomedical, Rodermark, Germany). Additionally, Abbott Inc. has incorporated creatinine detection into their i-STAT point-of-care system[10].

Potentiometric devices

Attempts at developing potentiometric biosensors for creatinine detection began as far back as 1976, with an ammonia-sensing electrode invented by Meyerhoff and Rechnitz[11]. Several years later, Guilbault and Coulet[12] developed a similar system with improved operational and storage stability. However, neither system was able to detect the lower reference range for creatinine and the long-term stability of these devices was still far short of commercial requirements. Mascini *et al.*[13] greatly extended the operational and storage stability of these gas-sensing devices, and reached the analytical range required to report normal, as well as pathological, specimens. Shin *et al.*[14] and Osaka *et al.*[15] have both published variations on the newly established potentiometric system.

To date, all potentiometric devices have been based on the catalysis of creatinine by creatinine iminohydrolase (CIH) at the surface of an NH_4^+-sensing ion-selective electrode. These systems have the advantage of relative simplicity because they require only a single enzyme and are based on well established gas-sensing electrode technologies; they also avoid interference from creatine. However, there are significant problems caused by interference from endogenous NH_4^+ in blood and, more significantly, in urine specimens. Recently, pre-analytical removal of endogenous ammonia has been reported[16], which uses the thermal decomposition of alkalinized urine samples with no detrimental effect on creatinine detection. However, the introduction of additional preanalytical steps is undesirable because it adds to the complexity of the assay and is a step away from the concept of analytical simplicity, which is fundamental to biosensor development.

Amperometric devices

With few exceptions, all amperometric biosensors for creatinine have been based on the three-enzyme method that was first described by Tsuchida and Yoda[17]. This involves the three-stage conversion of creatinine to creatine, creatine to sarcosine and sarcosine to glycine. In this final stage, consumption of electrochemically detectable oxygen and liberation of hydrogen peroxide (H_2O_2) occurs (Fig. 1).

The added complexity of a three-enzyme system has slowed down the development of these devices. and the presence of three enzymes means a loss in system sensitivity. Creatine potentially causes interference and dual sensors that measure and subtract creatine from creatinine are required, thus adding to system complexity, variability and error. Here, a sensor with creatine amidinohydrolase and sarcosine oxidase is used to

Anthony J.Killard (Tony.Killard@dcu.ie) and Malcolm R. Smyth (malcolm.smyth@dcu.ie) are at the National Centre for Sensor Research (NCSR), School of Chemical Sciences, Dublin City University, Dublin 9, Ireland.

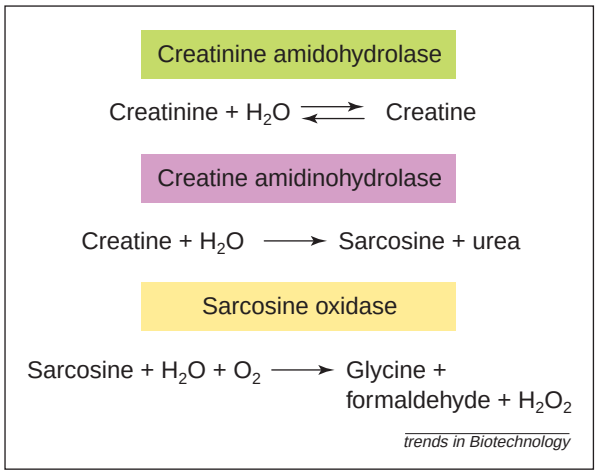

Figure 1

Three-stage enzymatic catalysis of creatinine to electrochemically detectable hydrogen peroxide (H_2O_2). Two sensors are usually required for the subtractive determination of creatinine from a combination of creatine and creatinine. The concentration of creatinine and creatine is determined using all three enzymes, whereas a second sensor that omits creatinine amidohydrolase, measures creatine. Thus, the creatinine concentration can be easily determined. The concentration of either creatine or creatine and creatinine is proportional to the amount of H_2O_2 generated, which is detected by reduction at the surface of an electrode.

calculate creatine levels in the specimen. However, these devices can achieve excellent operational stability and long storage lifetimes. In addition, other interferences (e.g. ammonia) are not a problem and nearly all devices achieve the analytical range required.

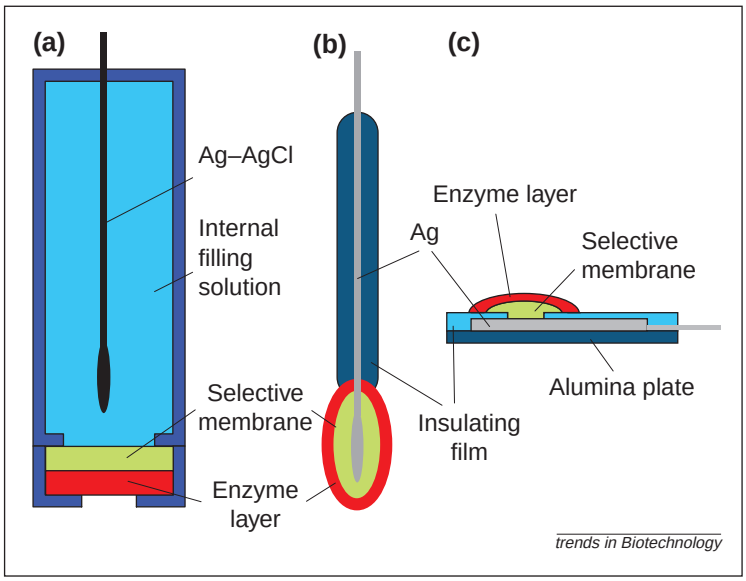

Figure 2

Potentiometric electrode configurations. (**a**) A conventional macroscale electrode (silver–silver chloride; Ag–AgCl) with an immobilized enzyme layer and a gas-sensitive membrane for the detection of ammonium (NH_4^+) or hydrogen (H^+) ions. (**b**) A similar configuration with Ag wire as the electrode. (**c**) Solid-state planar electrodes can be assembled on inert substrates such as alumina. An insulating layer is used to shield an Ag electrode, which is again modified with an immobilized enzyme layer and an inner selective membrane. All potentiometric creatinine electrodes are prone to interference from endogenous ammonia.

Detection of H_2O_2 liberation is the preferred technique in amperometric systems, although oxygen electrodes have also been used[18,19]. This preference is due to the classic problems associated with interference at oxygen electrodes (i.e. the high potentials required to bring about its reduction).

In both potentiometric and amperometric systems, several parameters are important in defining the sensitivity, analytical range, operational stability, storage stability and potential to prevent interference. These include: (1) the design of the underlying electrode surface; (2) the enzyme immobilization method and (3) the sensor configuration.

Electrode design

Potentiometric sensors for creatinine have used several well established configurations, including conventional macroelectrodes, wire and solid-state devices. Solid-state devices are based on thick- and thin-film technologies[15], which employ silver (Ag) or silver with silver chloride (Ag–AgCl) as the electrode material (Fig. 2). All recent amperometric creatinine sensors have been based on a platinum (Pt) electrode surface. Bare platinum electrodes[20], platinization of gold (Au) (Ref. 21) or shapable electroconductive (SEC) films[22], and carbon-paste electrodes mixed with platinum powder[23] have all been shown to be appropriate for creatinine sensor systems. Platinum has repeatedly been shown to have the best catalytic surface for the electrochemical oxidation of H_2O_2. It has been demonstrated that Pt deposited onto Au yielded the highest current for platinized SEC films[21,22]. No data was given for carbon-paste–Pt powder mixtures[23] or bare Pt surfaces[20]; however, all these devices (except the carbon-paste–Pt composite device) reach the required sensitivity for creatinine analysis. Such composites might be cost effective when compared to precious metal surfaces, but they still do not reach the operational criteria for creatinine measurement. At present, Pt would appear to be the preferred material for electrode fabrication.

Enzyme immobilization

The method of enzyme immobilization in both single enzyme and three-enzyme creatinine biosensors is the most important factor in the determination of their analytical range, and their operational and storage stability. Because stability remains one of the essential problems with these systems, a large variety of techniques has been investigated to find ways of producing sensors with commercial potential. These have included gel entrapment[20], polymer entrapment[15], crosslinking[22], direct nonimmobilized deposition[23] and combinations of these (Fig. 3). Most systems employ some form of crosslinking, which allows improved stability usually at the expense of decreased sensitivity and reduced analytical range.

Khan and Wernet[22] crosslinked the three enzymes directly onto the platinized SEC film in the presence of a gelatin film, thus reducing the exposure time of the enzymes to the glutaraldehyde crosslinker (although providing stability, glutaraldehyde crosslinking brings about significant reduction in enzyme activity). In this way, an excellent analytical range can be achieved (1 μM–5 mM), with the potential for continuous operation for 20–30 days. In addition, the presence of the gelatin layer reduced the diffusion of creatine to the

enzyme layer, thus reducing interference from this source. Reduction in responses to creatine could also be achieved by the optimization of the enzyme ratio. At 1 mM creatine, negligible responses resulted when the ratio of enzymes was 10:10:5 or 5:10:5 (mg ml^{-1} of sarcosine oxidase, creatine amidinohydrolase and creatinine amidohydrolase, respectively). A gradual increase in sensor response over a period of 18 days was attributed to the stabilization (swelling) of the gelatin matrix. Preswollen electrodes might be amenable to immediate use; however, no data were provided on the long-term stability of this system.

Stable entrapment of enzymes in a poly(carbomoyl) sulphonate-hydrogel matrix has been reported[20]. The hydrogel is formed using a bisulfide-blocked polyurethane prepolymer[24], which is deblocked with polyethylenimine. The reaction of isocyanate groups with polyol groups results in a hydrogel network in which the enzyme can be entrapped from the aqueous solution. This has led to the development of a biosensor with excellent long-term stability but a narrow analytical range (1–150 μM), which requires sample dilution before analysis.

Others have found that the crosslinking of the enzyme with glutaraldehyde, in the presence of bovine serum albumin (GA-BSA), is better than polymer entrapment in terms of operational stability and response time[21]. Entrapment in polyurethane hydrogel and poly-(2-hydroxyethylmethacrylate) matrices was compared with the GA-BSA method. Both polymer-entrapped systems show rapid drop-off in sensor response after only a few days, whereas the GA-BSA system shows excellent stability beyond three months.

Other strategies have been applied using polymers, such as polypyrrole or polyaniline, in single enzyme CIH biosensors of both potentiometrc[15] and amperometric[16] design. In the potentiometric system, an electrochemically inactive polypyrrole forms a polyion composite with CIH, using an H^+ electrode to detect changes in pH that are brought about by the enzymatic reaction; the limit of detection was quoted as 1 μM. The amperometric device employed a polyaniline–Nafion® composite onto which CIH was crosslinked (Fig. 4). The liberation of NH_4^+ was detected potentiometrically using an Ag–AgCl electrode. Although the system had a useful linear range of 0.5–500 μM, the problem of cation interference was significant. The electrode responded not only to endogenous NH_4^+ but also to other cations. However, it was stated that interference did not contribute more than 5% of the analytical signal when potassium, sodium, mercury, calcium, magnesium and aluminium ions were present individually at concentrations of 0.5 mM. A service life of ~3 months provides adequate long-term stability.

Direct deposition of enzymes has been reported (i.e. no specific immobilization procedure)[23]; however, no stability data has been provided and the method is unlikely to offer long-term stability.

Sensor configuration

The immobilization of enzymes at an electrode surface is rarely adequate to produce a device that meets all of the operational parameters required. The predominant problem is that of interference. Adding additional selective layers to a system can aid in reducing interference, but might also reduce the diffusion rates of substrates to the enzyme catalytic layer. This might not only reduce sensor sensitivity but can also extend the linearity of the system, potentially increasing the analytical range.

It has been shown that elements of the enzyme immobilization procedure can contribute to the perm-selectivity (the selective or semiselective movement of agents into or through a polymer matrix) of the sensor system[22]. However, additional complexity is often required to achieve suitable levels of selectivity. Madaras

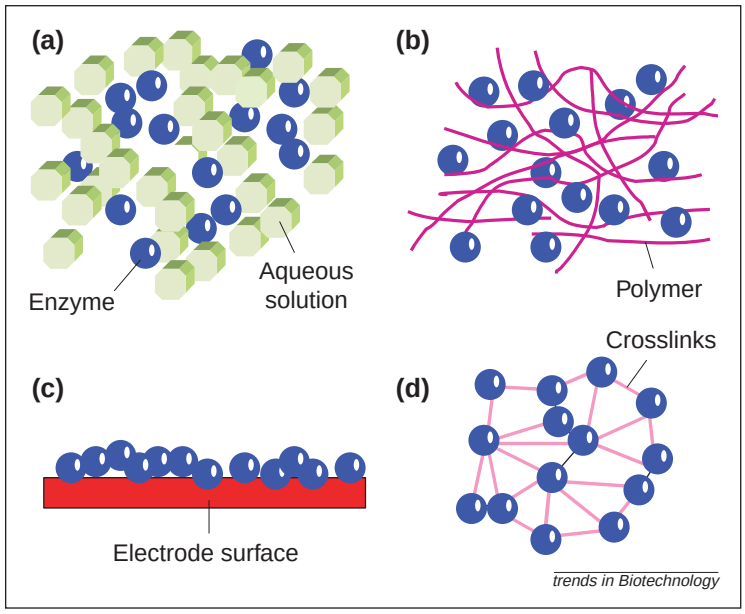

Figure 3

Basic methods of enzyme immobilization employed in creatinine biosensors. (**a**) Gel entrapment results in the enzyme being trapped in an aqueous environment. (**b**) Enzymes can be trapped within growing polymers. These can be either electroactive (e.g. polyaniline or polypyrrole), non-electroactive (e.g. Nafion®) or a combination of polymers. (**c**) Passive, noncovalent interactions with electrode surfaces have been used, but are unreliable. (**d**) Crosslinking of enzymes results in significant stability but this is often at the expense of enzyme activity, and thus sensitivity.

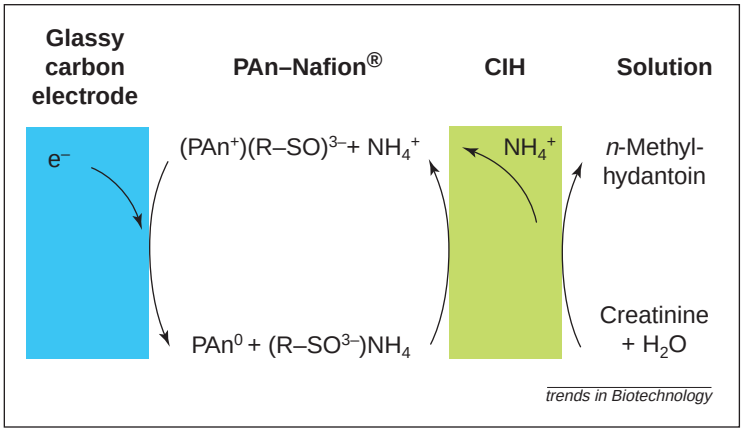

Figure 4

The amperometric measurement of creatinine using creatinine iminohydrolase (CIH) and a composite polyaniline–Nafion® polymer mixture (PAn–Nafion®). Liberation of ammonia causes the oxidation of the polymer composite, which is poised at a suitable potential to bring about its reduction. Reduction of the polyaniline produces a detectable current. (Reproduced, with permission, from Ref. 16.)

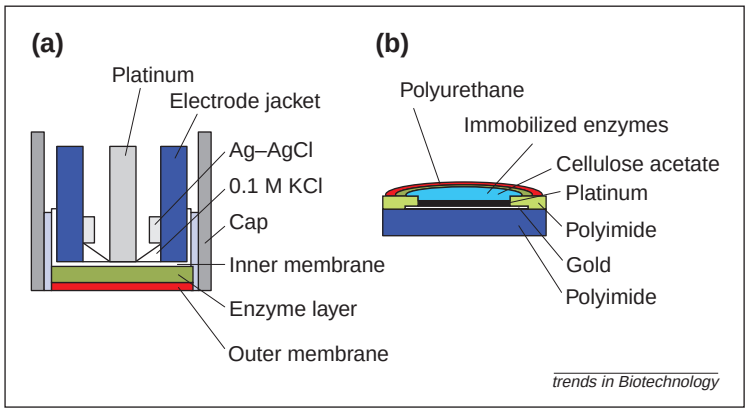

Figure 5

(**a**) Macro-scale and (**b**) microfabricated three-layer amperometric creatinine electrodes produced by Madaras *et al.* (Reproduced, with permission, from Ref. 21.) Both systems employ: (1) an outer membrane to act as a diffusional barrier (polycarbonate is the preferred choice for the outer membrane because good control of pore size can be achieved); (2) a middle enzyme layer; (3) an inner membrane composed of cellulose acetate for interference rejection. Use of three layers leads to increased stability and interference rejection, at the expense of dynamic range and sensitivity.

et al.[21] applied a three-layer strategy, using both an inner membrane layer adjacent to the electrode surface and an outer membrane layer; the crosslinked enzymes were entrapped between these layers. The performance of the inner membrane was evaluated and cellulose acetate was found to be superior in the rejection of interference (ascorbic acid and acetominophen) and adhesion properties when compared with Nafion® and other cellulose acetate mixtures (Fig. 5).

Various components were also investigated for the composition of the outer membrane to act as a diffusional barrier. Polycarbonate membranes were found to be difficult to apply to planar surfaces, although such membranes have excellent properties (e.g. the control of pore size). Compared with creatine, these membranes are highly permeable to oxygen, but allow faster substrate transfer to the enzyme layer.

In comparison with direct enzyme immobilization at the electrode, the systems of Madaras *et al.*[21], which employ inner and outer membranes, had significantly reduced sensitivities. Although the procedure was quoted as having a 30 μM limit for creatinine detection, the limit of quantification was not quoted. This might indicate that sensitivity in the three-layer system is reduced to a level that prevents analysis at the lower analytical range for creatinine. However, the combination of an inner permselective layer and an outer diffusional barrier gives the sensor excellent selectivity over all relevant interferences (ascorbic acid, acetominophen and creatine), improved linearity, and excellent operational and storage stability (at least three months).

Schneider *et al.*[20] also employed a sandwich arrangement for their hydrogel; they used either dialysis membranes or an outer polycarbonate membrane. However, the reduction in sensitivity was still ~50% and the upper analytical range for creatine was significantly higher than for creatinine (560 μM and 150 μM, respectively). Thus, creatine significantly interferes and the result is an analytical system that requires dilution before analysis. Further optimization of the character of both the permselective layers and the diffusional barriers is necessary to achieve the desired balance of selectivity, sensitivity and interference rejection.

Shin *et al.*[14] used asymmetric membranes of hydrophilic polyurethane mixed with enzyme, coated onto a polyurethane ion-selective membrane for NH_4^+. However, apart from the fact that the properties of the polyurethane can vary widely between sources, no information was given about the long-term stability of these devices.

Conclusions

Although possessing certain unique features (e.g. enzymatic pathways, and the nature of interferences such as creatine and NH_4^+), the development of electrochemical creatinine biosensors is representative of developments in the wider world of biosensor research. This article has attempted to show how the various elements of a sensor dictate the performance parameters of the device. It also attempts to show how optimization in electrode design, enzyme immobilization and overall sensor configuration have gradually resulted in viable creatinine biosensors; most notably, three-layer systems appear to provide the best balance of sensitivity, stability and selectivity. Although problems associated with these parameters still exist, creatinine sensors are now meeting their full commercial potential. In recent years, research focus has switched to amperometric systems; however, potentiometric devices still have great potential and should not be ignored, particularly when the difficulties of interference are overcome. Amperometric devices using either a single enzyme system or a three-enzyme system with better rejection of creatine interference, will also simplify these devices by removing the requirement for subtractive measurements from two electrodes. These developments will ultimately lead to their widespread clinical application.

References

1 Bakker, E. *et al.* (1999) Ion sensors: current limits and new trends. *Anal. Chim. Acta* 393, 11–18
2 Tietz, N.W. (1986) In *Textbook of Clinical Chemistry* (1st edn), p. 1810, Saunders
3 Sena, F.S. *et al.* (1988) Effect of high creatinine content on the Kodak single-slide method for creatinine. *Clin. Chem.* 34, 594–595
4 Madaras, M.B. and Buck, R.P. (1996) Miniaturized biosensors employing electropolymerized permselective films and their use for creatinine assays in human serum. *Anal. Chem.* 68, 3832–3839
5 Jaffé, M. (1886) Z. *Physiol. Chem.* 10, 391–400
6 Siedel, J. *et al.* (1984) Sensitive color reagent for the enzymatic determination of creatinine. *Clin. Chem.* 30, 968–969
7 Weber, J.A. and van Zanten, A.P. (1991) Interferences in current methods for measurement of creatinine. *Clin. Chem.* 37, 695–700
8 Jeppesen, M.T. and Hansen, E.H. (1988) Determination of creatinine in undiluted blood serum by enzymatic flow injection analysis with optosensing. *Anal. Chim. Acta* 214, 147–159
9 Killard, A.J. *et al.* (2000) Electroanalysis and biosensors in clinical chemistry. In *The Encyclopedia of Analytical Chemistry* (Vol. 5) (Meyers, R., ed.), pp. 173–207. John Wiley & Sons, NY, USA
10 Mock, T. *et al.* (1995) Evaluation of the i-STAT™ system – a portable chemistry analyser for the measurement of sodium, potassium, chloride, urea, glucose, and hematocrit. *Clin. Biochem.* 28, 187–192
11 Meyerhoff, M. and Rechnitz, G.A. (1976) An activated enzyme electrode for creatinine. *Anal. Chim. Acta* 85, 277–285
12 Guilbault, G.G. and Coulet, P.R. (1983) Creatinine-selective enzyme electrodes. *Anal. Chim. Acta* 152, 223–228
13 Mascini, M. *et al.* (1985) Ammonia abatement in an enzymatic flow system for the determination of creatinine in blood sera and urine. *Anal. Chim. Acta* 171, 175–184

14 Shin, J.H. *et al.* (1998) Potentiometric biosensors using immobilized enzyme layers mixed with hydrophilic polyurethane. *Sens. Actuat. B* 50, 19–26

15 Osaka, T. *et al.* (1998) Highly sensitive microbiosensor for creatinine based on the combination of inactive polypyrrole with polyion complexes. *J. Electrochem. Soc.* 145, 406–408

16 Shih, Y-T. and Huang, H-J. (1999) A creatinine deiminase modified polyaniline electrode for creatinine analysis. *Anal. Chim. Acta* 392, 143–150

17 Tsuchida, T. and Yoda, K. (1983) Multi-enzyme membrane electrodes for determination of creatinine and creatine in serum. *Clin. Chem.* 29, 51–55

18 Kubo, I. *et al.* (1983) Amperometric determination of creatinine with a biosensor based on immobilized creatininase and nitrifying bacteria. *Anal. Chim. Acta* 151, 371–376

19 Nguyen, V.K. *et al.* (1991) Immobilized enzyme electrode for creatinine determination in serum. *Anal. Chem.* 63, 611–614

20 Schneider, J. *et al.* (1996) Hydrogel matrix for three enzyme entrapment in creatine/creatinine amperometric biosensing. *Anal. Chim. Acta* 325, 161–167

21 Madaras, M.B. *et al.* (1996) Microfabricated amperometric creatine and creatinine biosensors. *Anal. Chim. Acta* 319, 335–345

22 Khan, G.F. and Wernet, W. (1997) A highly sensitive amperometric creatinine sensor. *Anal. Chim. Acta* 351, 151–158

23 Kim, E.J. *et al.* (1999) Disposable creatinine sensor based on thick-film hydrogen peroxide electrode system. *Anal. Chim. Acta* 394, 225–231

24 Vorlop, K.D. *et al.* (1992) Entrapment of microbial cells within polyurethane hydrogel beads with the advantage of low toxicity. *Biotechnol. Tech.* 6, 483–488