Selective and sensitive electrochemical biosensing of superoxide anion production by biological systems: a short overview of recent trends

M. Pontié and F. Bedioui

École Nationale Supérieure de Chimie de Paris, Laboratoire d'Électrochimie et de Chimie Analytique (UMR CNRS-ENSCP n° 7575), 11, rue Pierre et Marie Curie, 75231 Paris Cedex 05, France

Biologically, superoxide anion (O_2^-) has been proposed to be directly or indirectly involved as a signaling messenger in vascular dysfunction. Measuring the local concentrations of this free radical in biological models is very difficult because of its high reactivity and fleeting existence. We report in this review selected significant examples of electrochemical micro electrodes and biosensors especially and appropriately designed for specific *in situ* determination of superoxide O_2^- in biological systems. This report will address and illustrate the desirable characteristics and the diverse ranges of the fabrication of the electrochemical biosensors and introduce the readers to some significant examples of their applications in this field.

Introduction

Biologically, superoxide anion (O_2^-) has been proposed to be directly or indirectly involved as a signaling messenger in vascular dysfunction [1,2]. An overproduction has been observed during ischemia and reperfusion and in pathologies such as hypercholesterolemia, atherosclerosis and diabetes. The superoxide anion radical belongs to reactive oxygen species and may damage organisms if it exceeds the level at which these organisms are able to provide defence. It is also known that several types of cells, including endothelial cells and immuno-defense cells such as macrophages and neutrophils, can produce simultaneously nitric oxide (NO) and O_2^- [3,4]. It is thus essential for the understanding of their role in cell functions to be able to follow simultaneously their production. However, there is limited knowledge about

Article available at http://analusis.edpsciences.org or http://dx.doi.org/10.1051/analusis:1999270564

their *in situ* simultaneous production by vascular cells. In spite of the major interest in evaluating the balance between nitric oxide and O_2^- production, this approach has so far been hampered by the difficulty to dispose of non-perturbing methods to quantify the local concentrations of these biological mediators.

The biological production and reactivity of NO are now largely studied. This has been allowed by the possibility to measure directly its concentration, particularly by electrochemical methods [5,6]. The quantification of the production of superoxide anions is hard to achieve because of their natural and/or catalyzed disproportionation by superoxide dismutase [7] and of their high reactivity towards other small molecules [2,8,9]. It appears that measuring O_2^- in biological models is very difficult because of its low concentration and fleeting existence. Its determination *in vivo* is a topical problem in clinical medicine.

Most of the techniques for assaying O_2^- release use indirect spectroscopic methods [10]. In particular:

– spectrophotometric measurement of the amount of ferricytochrome *c* reduced by O_2^-, according to the following reaction:

cytochrome *c* (Fe^{III}) + O_2^- → cytochrome *c* (Fe^{II}) + O_2

since ferrocytochrome *c* exhibits a strong absorbance at 550 nm [11],

– chemiluminescence method which is based on the measurement of the intensity of the fluorescence radiation emitted after chemical oxidation of O_2^- by lucigenin [12,13],

– electron spin resonance spectroscopy [14].

These three strategies are now widely developped and provide more or less efficient measurements of O_2^- in biological samples. Nevertheless, these strategies suffer from being *ex situ* detection techniques with poor selectivity or sensitivity (the chemiluminescence technique is the most sensitive, with a O_2^- detection limit of 2×10^{-8} M, compared to 5×10^{-6} M with the ESR technique).

The desire to selectively measure small amounts of O_2^- release *in situ* has led to an active area of research involving the design of micro sensors. In fact, one of the challenges in developing micro sensors is the necessity to achieve *in vivo* real-time O_2^- detection in intact tissues or from single cells. New amperometric microelectrode probes are now developed to detect O_2^- and the use of electrochemistry as a potential way to do so is very promising. Indeed, such a strategy offers specific advantages, namely *i*) a minimal alteration of the concentration to be measured; *ii*) a high selectivity that allows to avoid chemical or enzymatic interventions, and *iii*) the possibility to follow at the sub-second level its local time-dependent change. In addition, micro electrode design and fabrication are now reaching very high levels of sophistication [15,16] which actively contributes to promoting the use of electrochemical techniques for *in vivo* O_2^- determination.

An especially fruitful strategy for reaching highly specific and selective electrochemical sensing of O_2^- is the use of specific enzyme-based biosensors, namely cytochrome *c* or superoxide dismutase (SOD). In this *review* we will address recently published works that have involved micro biosensors or simple chemically modified micro electrodes specially designed for the electrochemical detection of O_2^- in biological media.

Amperometric Biosensors: designs and applications

Cytochrome c – based biosensor

The most developed device is constituted of cytochrome *c*-coated gold [17-27] or platinized carbon electrode [21,28,29]. Superoxide anion reduces the immobilized ferricytochrome *c*, which is immediately re-oxidized by the surface-modified electrode at a potential of 15-25 mV *vs* Ag/AgCl, as illustrated in figure 1.

The manufacturing of the cytochrome *c*-coated gold electrodes proceeds usually as follow [17,18]: the surface of the gold disc electrode (diameter 1.5-4 mm) is first modified by immersion in 2 mM *N*-acetyl cysteine phosphate buffer solution (10 mM, pH = 7) for two hours and cytochrome *c* is then covalently immobilised at the electrode surface via peptide coupling by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide to initiate the condensation reaction (for six hours at 4 °C). Promoters other than *N*-acetylcysteine (*i.e* .4-4-dithiobipyridine [22] or 3,3-dithiobis(sulphosuccinimidyl-propionate [19, 23, 24, 30]) can also be used. Indeed, in order to have an efficient electron transfer between the supported enzyme and the electrode surface, a very well orientation of the cytochrome is needed. This issue is generally addressed by coating the electrode surface by promoters with specific functional groups able to interact and to orientate specifically the enzyme macromolecules, which makes possible a fast electron transport from the redox protein to the electrode.

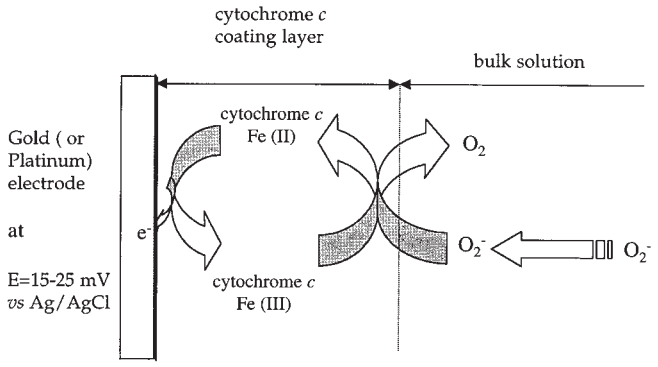

Figure 1. General sequence of reactions for the cytochrome *c*-based biosensors (adapted from ref. [17])

Another approach for making cytochrome c-coated electrodes consistes to achieve the immobilisation of cytochrome *c* by passive adsorption on platinized carbon electrode, PACE, as follow: a platinized activated carbon electrode (1 cm^2 area) is immersed in a phosphate buffer solution (pH = 7.4) of cytochrome *c* (10 mg/ml) and shaked gently for few hours [21]. After this time, the PACE incorporating adsorbed cytochrome *c* is rinsed throughly and stored in phosphate buffer solution at 5°C.

The sensitivity of these both biosensors to the superoxide anion was generally assessed by using the xanthine (Xa) / xanthine oxidase (XOD) reaction:

$$Xa + O_2 + 2H_2O + XOD \rightarrow \text{uric acid} + 2H^+ + 2O_2^-$$

in 20 mM phosphate buffer solution (pH = 7) at room temperature. The reaction is usually initiated by the addition of XOD in the presence of saturating or 0.5 mM Xa [17,22,23,25], to give a final concentration enzyme in the range 0-1 µM [17,18,21,23,30]. The sensitivity of the cytochrome *c*-coated gold electrode, defined as the rate of change in current density per unit concentration of XOD, was calculated as being equal to 2.05 $\mu A.cm^{-2}.min^{-1}.\mu M^{-1}$ and the sensitivity of the PACE electrode was evaluated as being equal to 34.0 $\mu A.cm^{-2}.min^{-1}.\mu M^{-1}$ [21]. The increase in sensitivity for the PACE configuration is thought to arise from the much larger effective surface area of PACE compared with the planar gold electrode. Indeed, PACE is an extremly porous material capable of binding much larger amounts of cytochrome *c* than a gold electrode of the same diameter.

The specificity of these biosensors was confirmed in few cases, but it should be noted that the biosensor specificity is directly linked to that of the reoxidation current, which constitutes the analytical signal. This latter is not only dependent on the supposed specificity of cytochrome *c* reduction by superoxide anions, but may also be dependent on the presence of other reducing or oxidizing constituents in the medium. Finally, it has been found that the PACE electrodes have a lifetime in use of about 6h.

Campanella and coworkers have recently reported the use of a carbon paste electrode configuration having cytochrome *c* and Fe(III)-protoporphyrin as a redox mediator [31]. The carbon paste electrode is made of cellulose triacetate gel of 30 mm of diameter and 0.5 mm width which is compressed with 125 mg of graphite powder and 50 µL of paraffin oil. The used of Fe(III)-proptoporphyrin IX is aimed at reoxidating the reduced ferricytochrome *c*. In this case, the oxidation current of the Fe(II) form of the protoporphyrin IX constitutes the analytical current for continuous superoxide anion determination at applied constant potential of 0.8 V *vs* saturated calomel electrode (SCE). A response time of 2 minutes and a long lifetime of 3 days were reported along with a very high detection limit of 0.2 mM. These characteristics must be improved for an accurate development of this biosensor configuration in biological systems.

As far as the biological applications of the above described biosensors are concerned, the most developed one is the cytochrome *c*-coated gold electrode because of its easy build. Several applications has been reported in the literature, such as:

- the development of a mathematical model for the O_2^- biosensor responding to enzymatically produced O_2^- which should enable the determination of absolute concentrations of superoxide anion in biological systems when the electrode is employed for direct, real-time monitoring of free radical release and interactions [30];
- the measurement of free-radicals production and cellular activity in disease processes where free radical species act as mediators which cause and perpetuate inflammation in disease states, including rheumatoïd arthritis and neurodegenerative disorders [25];
- the detection of O_2^- generation by isolated mammalian osteoclasts that can be used as an index of the osteoclast activity [23,25];
- the elucidation of a novel function for the astrocytic constitutive nitric oxide synthase in regulating extracellular superoxide release and, therefore, controlling neuronal nitric oxide availability [20];
- the understanding of the biochemical and molecular mechanisms of nerve cell death and the role of O_2^- and NO radicals in cell mortality [19];
- the determination of SOD activity entrapped into liposomes and the study of the interference from hydrogen peroxide and uric acid [27].

In the case of the PACE biosensor, the reported applications are especially related to the demonstration of traumatic brain injuries resulting in increases in superoxide anion production [28] and the detection of O_2^- generated by the brain during hypoxia/hypercarbia, focal ischemia and various brain injuries in the rat [29]. PACE was also demonstrated being the most sensitive and the most suitable for use in combination with stimulated human neutrophils [21].

Superoxide dismutase (SOD) – based biosensors

Mesaros and coworkers [32] described the design of an amperometric biosensor based on the use of a platinum wire covered with electropolymerized pyrrole containing SOD. The biosensor was prepared by anodic polymerization of pyrrole and concomittant incorporation of SOD on a Pt wire in phosphate buffer solution. First, the surface of the cylindrical Pt microelectrode (with a radius of 20 µm and a length of 6 mm) was polished electrochemically in 1 M H_2SO_4, using cycling voltammetry from –1 to 1 V *vs* SCE, and then rinsed with distilled water. To incorporate superoxide dismutase into the conducting polypyrrole film, electropolymerization was conducted at 0.8 V *vs* SCE using controlled potential coulometry at the Pt microelectrode from degassed phosphate buffer solution (pH=7.4) containing p-tosyl ions as supporting electrolyte, pyrrole as monomer and the enzyme SOD. The prepared enzyme electrode was stored in phosphate buffered saline (pH=7.4), at 4°C when not used.

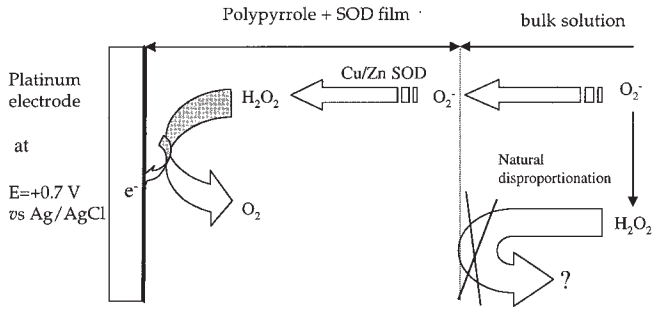

Figure 2. General sequence of reactions for the polypyrrole + SOD-based biosensor (adapted from ref. [32]).

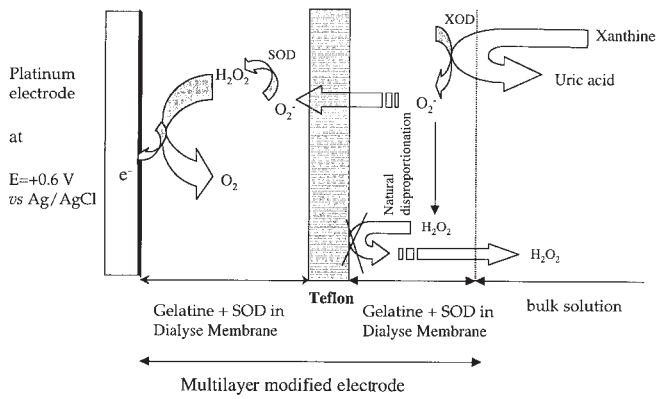

Figure 3. General sequence of reactions for the multilayered Teflon and SOD-containing gel biosensor (adapted from ref. [33]).

The way of working of the above described SOD-based biosensor is shown in figure 2: the superoxide anion penetrates from the bulk solution into the polypyrrole film, where it is disproportionated by SOD to hydrogen peroxide. The generated hydrogen peroxide is then oxidized at the electrode surface at 0.7 V/SCE. Thus, the amperometric response of this biosensor is directly linked to the anodic oxidation of the generated hydrogen peroxide inside the polypyrrole film, which constitutes an indirect way of evaluating the superoxide production within the solution external to the polymer coating. The performances of this biosensor were evaluated by using enzymatically generated superoxide anion by the Xa/XOD reaction. They were reported as being very good because of the low detection limit (15 nM), the optimal pH which is very close to the biological of pH 7.4, the easy preparation, the short response time (<5s) and the temperature stability. But it is unclear from the reported experiments whatever or not this biosensor allows to distinguish between hydrogen peroxide produced within the polypyrrole film and hydrogen peroxide produced in the external solution, by natural disproportionation of O_2^- and diffusing from the solution to the film.

One should also mention the use of SOD-coated PACE electrode (diameter 1.5 mm) developped by McNeil and coworkers [21]. Bovine Cu/Zn SOD was immobilised on PACE by using an identical method to that described for cytochrome *c*. The SOD-coated biosensor was polarized at +320 mV *vs* Ag/AgCl to estimate H_2O_2 produced by the enzymic disproportionation of O_2^- [22], through its oxidation current. Since there are two sources of H_2O_2 that hence the current in this system (that from the SOD disproportionation reaction and that from the natural disproportionation reaction), it was therefore necessary to substract from the combined current that due to the natural disproportionation reaction alone. This was measured and eliminated by using a second electrode consisting of bovine serum albumin-coated PACE electrode in conjuction with the bipotentiostat poised at +320 mV *vs* Ag/AgCl.

Finally, Song and coworkers [33] have reported the design of an amperometric superoxide biosensor based on the use of an SOD-containing gel which is placed on a dialysis membrane and covered with an external Teflon membrane. The authors showed that the outer Teflon membrane is able to descriminate between superoxide radicals and endogeneous hydrogen peroxide and other interfering compounds. The evaluation of the performances of this biosensor was achieved by using a XOD layer which was placed onto the Teflon membrane and covered by a dialysis membrane (see figure 3). The sandwich layers were placed in front of a platinum electrode (0.5 mm diameter) as working electrode, which was polarized at 0.6 V *vs* Ag/AgCl. This gel entrapped SOD is able to converted a source of O_2^- (by using Xa/XOD reaction, with Xa in solution while XOD is entrapped in a gel layer as shown in figure 3) to H_2O_2 which is oxidized at 0.6 V.

Few applications of these SOD-based biosensors were detailed in the literature. We can mention the use of the polypyrrole/SOD biosensor in the simultaneous NO and O_2^- determination in a cardiovascular system (abdomina aorta of dog) after stimulation by agonists [34], the use of PACE/SOD biosensor for the evaluation of superoxide production by human neutrophils [21] and the possible application of the Teflon/SOD biosensor configuration in homogeneous immunoassays [33].

Horseradish peroxidase (HRP) and SOD – based biosensors

Lvovich and Sheeline [35] described an elegant way for building a two-channel biosensor configuration based on the simultaneous presence of horseradish peroxidase (HRP) and SOD embedded in a polypyrrole layer on a micro glassy carbon electrode (1 mm diameter). Superoxide generated by the interaction of xanthine and xanthine oxidase, or by injection of KO_2 at basic pH, is initially disproportionated by SOD. Then sensed electrode current at +0 mV *vs* NHE resulted from HRP-mediated reduction of enzymatically generated hydrogen peroxide, or hydrogen peroxide from bulk solution which diffuses through the SOD layer. Thus,

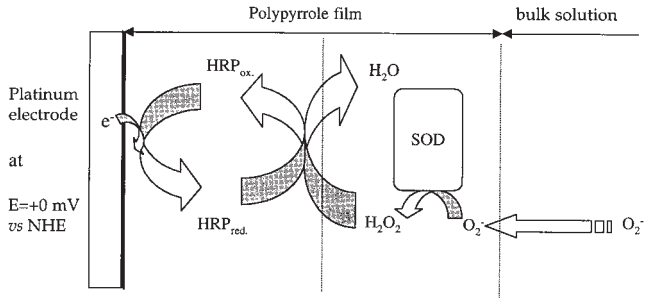

Figure 4. General sequence of reactions for the two channel HRP and SOD-based biosensor (adapted from ref. [35]).

the mechanism of this biosensor is based on redox chemistry, leading to regeneration of native peroxidase by current supplied by the electrode (Fig. 4).

The reported properties of this amperometric biosensor are the following [35] detailed in the figure 4: it is a compact system with a calibration range of 5×10^{-8}-10^{-6} M and a sensitivity (defined as the rate of change of current density per unit concentration of XOD) of 0.114±0.006 $A.cm^{-2}.M^{-1}$ (with KO_2 the sensitivity is double). The sensitivity of the biosensor for H_2O_2 is 0.266±0.008 $A.cm^{-2}.M^{-1}$ (n=25) and is independent of pH variations. It works with a good stability during 10 days at room temperature (7% decrease in sensitivity in a week, better than a refrigerated one which shows 30% decrease).The superoxide sensor sensitivity to XOD injections was found to be 0.059±0.003 $A.cm^{-2}.M^{-1}$.

There is only one reported application for this bi-enzymatic sensor. It is related to the detection of H_2O_2 and O_2^- for a better understanding of the peroxidase/NADH oscillator [36].

Non-enzymatic amperometric sensors

It is important to mention the various approaches reported in the literature and based on the direct electrochemical oxido-reduction of superoxide radicals (or their derivatives) on non-enzymatically modified electrodes. For example, Darmon and coworkers [37] reported an indirect approach in sensing superoxide anions by using a Clark's type electrode at which O_2^- was indirectly measured through its transformation to O_2. But this kind of electrode is not designed to be applicable in biological media. Ohsaka and coworkers [38] reported the use of a hanging mercury drop electrode (with and without coating by a molecular hydrophobic compound such as alpha-quinoline) to investigate O_2^- redox behavior and examinate a variety of biomolecular reactivities of O_2^- in aqueous media. The presence of the hydrophobic alpha-quinoline adsorbed on the surface of the hanging mercury drop electrode enables the authors to observe, on the usual time scale of cyclic voltammetry, the one electron process of the O_2/O_2^- redox couple even in aqueous media.

Recently, Campanella and coworkers [39] reported a new method for radical determination based on spin trapping reaction suitably adapted for the electrochemical determination of superoxide and hydroxide radicals. It consisted in the use of benzylidenephenylnitrone immobilized in a PVC-sebacate membrane deposit (0.2 mm of thickness) on a glassy carbon electrode or as a ChemFET (chip dimension of 1.28 × 2.16 mm). The way of working of this membrane sensor is based on the peculiar properties of benzylidenephenylnitrone which can generate adducts when it reacts with radicals. This membrane has been used in the assembly of different kinds of selective potentiometric sensors and field effect transistor devices for superoxide and hydroxide radical determination. The classical potentiometric selective membrane sensor gives a linear response in the range 0.2-1.7 mM for superoxide radicals and in the range 0.3-1.8 mM for hydroxide radicals, while the sensor based on the field effect transistor gives a linear response in the range 25 mM-1.0 mM for superoxide radicals. But the poor detection limits of these devices (0.03 mM) eliminate their potential use in biological applications.

The more efficient reported way in measuring *in situ* biological production of superoxide radicals by direct oxidation on non-enzymatic electrodes were based on building carbon microfibers [8, 40-44] with micrometric dimensions. The carbon fiber is sealed in a glass capillary by heating the glass to melt it around the fibre [40] or by using epoxy resin [8, 41, 42, 44]. The electrode was then polished prior to each run [40] or electrochemically pretreated [8,41]. One should recall that there are two main advantages to the degree of miniaturisation necessary for implantation of the O_2^- electrode in biological media. First, as a direct consequence of the reduced geometry, the electrode will experience hemispherical diffusion mass transport characteristics which, with associated short diffusion lengths, implies that responses will be fast : for example the diffusion coefficient for O_2^- is 10^{-5} $cm^2.s^{-1}$, indicating that O_2^- microelectrodes will have a steady-state response time of less than 1 s. Secondly, and as a consequence of the very small volume surrounding cells, when the electrode is accurately positioned, messenger flux to or from cells will give local high concentration in the environment close to the sensor.

The first direct electrochemical characterization of O_2^- concentration was reported by Tanaka and coworkers [40]. It was based on the oxidation of O_2^- at a carbon microelectrode set at 0.1 V *vs* SCE and it has allowed to demonstrate periodic fluctuations in O_2^- production by a single phagocytic cell, adsorbed on the opsonized electrode [42]. This study showed a new technique for bringing a single neutrophil cell into a contact with the surface of a microelectrode under the microscope. The authors also showed successful trials in detecting O_2^- from a single living cell [44] and from HL-60 cells differenciated with retinoïc acid which were confirmed to act as a neutrophils and generated O_2^- when stimulated [43, 44].

The most recent trends of applications of the direct oxidation of superoxide anions on microfiber electrodes at 0.12V *vs* SCE, is related to the double determination of O_2^- and NO and the direct and simultaneous electrochemical

measurements of both nitric oxide and superoxide anion evolution and reactivity in solution [8]. The use of the NO sensor provides the selective detection of NO without any interference of the superoxide enzymatic generator [8]. More recently, Privat and coworkers [41] described the first report of direct real time monitoring of extracellular O_2^- production in stimulated human vascular cells by this electrochemical method. Indeed, release of O_2^- by cultured vascular cells was investigated with the use of the selective microelectrode, in the range of 10^{-8} M whithout interference of the major oxygen species and with an acceptable sensitivty of 1.65±0.42 pA/nM for O_2^- (n=6). It should be emphasized that low interferences were observed due of the low working oxidation potential of the electrode (0.12V *vs* SCE) and the differential pulse amperometric technique. But, possible problems may be associated with the *in vivo* use of these electrodes such as protein fouling and subsequent loss of the electrochemical response. These complications may be resolved by an effective protection of the modified electrode surface, by developing perm-selective membranes without decreasing too much the sensor sensitivity [45].

Conclusion

It appears from studies of the electrochemical determination of superoxide radicals described above that the idea of designing microsensor devices with specific behavior towards O_2^- is realistic. The use of the enzymatically modified micro electrode approach provides an elegant way to build up multilayered structures and architectures that result in superoxide amperometric biosensors with high performance characteristics. In most cases, the selectivity of the biosensors is largely improved through use of enzymes. It is also important to give special attention to the electrochemically pretreated carbon microsensors which appear to be powerful new analytical tools. Our group's successful fabrication, calibration and use of the carbon microfiber electrodes for biological superoxide radicals determination established the potential future development of this class of amperometric sensors. In a general way, the significant developments in new enzymatic materials combined with the explosive growth in biosensor science and technology should stimulate intense exploration of this field of research.

References

1. Suzuki, Y.J.; Forman, H.J.; Sevanian, A. *Free Radic. Biol. Med.* **1997**, *22*, 269-285.
2. Finkel, T. *Curr. Opin. Cell. Biol.* **1998**, *10*, 248-253.
3. Squadrito, G.L.; Pryor, W.A. *Free Radic. Biol. Med.* **1998**, *25*, 392-403.
4. Beckman, J.S.; Crow, J.P. *Biochem. Soc. Transactions* **1993**, *21*, 330-334.
5. Malinski, T.; Czuchajowski In: *Methods in Nitric Oxide Research* (Feelisch M, Stamler JS, eds.); J. Wiley & Sons Ltd: Chichester, 1996, pp 319-339.
6. Bedioui, F.; Trévin, S.; Devynck, J. *Electroanalysis* **1996**, *8*, 1085-1091.
7. Vandewalle, P.L.; Petersen, N.O.; *FEBS Lett.* **1987**, *210*, 195-198.
8. Privat, C.; Trévin, S.; Bedioui, F.; Devynck, J. *J. Electroanal. Chem.* **1997**, *436*, 261-265.
9. Carmichael, A.J.; Steel-Goodwin, L.; Gray, B.; Arroyo, C.M. *Free Radic. Res. Commun.* **1993**, *19*, S1-S16.
10. Fridovich, I. *J. Biological Chem*. **1997**, *272*, 18515-18517.
11. Koppenol, W.H.; van Buuren, K.J.; Butler, J.; Braams, R. *Biochim. Biophys. Acta* **1976**, *449*, 157-168.
12. Gylenhammar, H. *J. Immunol. Methods* **1987**, *97*, 209-213.
13. Ohara, Y.; Peterson, T.E.; Harrison, D.G. *J. Clin. Investig*. **1993**, *91*, 2546-2551.
14. Harbour, J.R.; Hair, M.L. *J. Phys. Chem*. **1978**, *82*, 1397-1399.
15. *Biosensor Technology : Fundamentals and Applications* (Buck, R.; Hatfield, W.E.; Umana, M.; Bowden, E.F. eds); Marcel Dekker: NY, 1990.
16. Bond, A.M. *Analyst* **1994**, *119*, R1-R13.
17. Cooper, J. C.; Thompson, G.; McNeil, C. J. *Mol. Cryst. liq. Cryst.* **1993**, *235*, 127-132.
18. Cooper, J. C.; Greenough, K. R.; McNeil, C. J. *J Electroanal Chem.* **1993**, *347*, 267-275.
19. Manning, P.; McNeil, C. J.; Cooper, J. M.; Hillhouse, E. W. *Free Radical Biol. Med.* **1998**, *24*, 1304-1309.
20. Tolias, C. M.; McNeil, J. C.; Kazlauskate, J.; Hillhouse, E. W. *Free Radical Biol. Med.* **1999**, *26*, 99-106.
21. McNeil, C. J.; Greenough, K. R.; Weeks, P. A.; Self, C. H. *Free Rad. Res. Comm.* **1992**, *17*, 399-406.
22. McNeil, C. J.; Smith, K. A.; Bellavite, P.; Bannister, J. V. *Free Rad. Res. Comm.* **1989**, *7*, 89-96.
23. Datta, H. K.; Rathod, H.; Manning, P.; Turnbull, Y.; McNeil, C. J. *J. Endocrinology* **1996**, *149*, 269-275.
24. Datta, H. K.; Manning, P.; Rathod, H.; McNeil, C. J. *Experimental Physiology* **1995**, *80*, 713-719.
25. McNeil, C. J.; Athey, D.; Ho, W. O. *Biosensors & Bioelectronics* **1995**, *10*, 75-83.
26. Tammaveski, K.; Tenno, T. *Proc. Estonian Acad. Sci. Chem.* **1995**, *44*, 156-164.
27. Lisdat, F.; Ge, B.; Ehrentreich-Förster, E.; Reszka, R.; Scheller, F. W. *Anal. Chem*. **1999**, *71*, 1359-1365.
28. Fabian, R. H.; DeWitt, D. S.; Kent, T. A. *J. Neurotroma* **1998**, *15*, 433-440.
29. Fabian, R. H.; DeWitt, D. S.; Kent, T. A. *J. Cerebral Blood Flow Metabolism* **1995**, *15*, 242-247.
30. Tammaveski, K.; Tenno, T.; Mashirin, A. A.; Hillhouse, E.W.; Manning, P.; McNeil, C. J. *Free Radical Biol. Med.* **1998**, *25*, 973-978.
31. Campanella, I.; Favero, G.; Tomassetti, M. *Sensors & Actuators B* **1997**, *44*, 559-565.
32. Mesaros, S.; Vankova, Z.; Grunfeld, S.; Mesarosova, A.; Malinski, T. *Anal. Chim. Acta* **1998**, *358*, 27-33.
33. Song, M.I.; Bier, F. F.; Scheller, F. W. *Bioelectrochem. & Bioenerg*. **1995**, *38*, 419-422.
34. Mesaros, S.; Vankova, Z.; Mesarosova, A.; Tomeik, P.; Grunfeld, S. *Bioelectrochem. & Bioenerg*. **1998**, *46*, 33-37.
35. Lvovich, V.; Scheeline, A. *Anal. Chem*. **1997**, *69*, 454-462.

36. Lvovich, V.; Scheeline, A. *Anal. Chim. Acta* **1997**, *354*, 315-323.

37. Darmon, N.; Fernandez, Y.; Periquet, A.; Mitjavila, S. *Free Rad. Res. Comm.* **1992**, *17*, 97-107.

38. Ohsaka, T.; Matsumoto, F.; Tokuda, K. In: *Frontiers of Reactive Oxygen Species in Biology and Medicine* (Asada K, Yoshikawa T, eds.); Elsevier Science B.V.: Oxford, 1994, pp 91-93.

39. Campanella, I.; Favero, G.; Occhienero, F.; Tomassetti, M. *Analusis* **1998**, *26*, 223-228.

40. Tanaka, K.; Kobayashi, F.; Isogai, Y.; Iizuka, T. *Bioelectrochem. & Bioenerg.* **1991**, *26*, 413-421.

41. Privat, C.; Stepien, O.; David-Dufilho, M.; Brunet, A.; Bedioui, F.; Marche, P; Devynck, J.; Devynck, M.-A. *Free Radicals Biol. Med.* **1999**, in press.

42. Isogai, Y.; Tsuyama, T.; Osada, H.; Iizuka, T.; Tanaka, K. *FEBS Letters* **1996**, *380*, 263-266.

43. Tanaka, K.; Tsuyama, T.; Isogai, Y.; Iizuka, T. In: *Frontiers of Reactive Oxygen Species in Biology and Medicine* (Asada K, Yoshikawa T, eds.); Elesevier Science B.V.: Oxford, 1994, pp 89-90.

44. Tanaka, K.; Tsuyama, T.; Karatsu, Y.; Iizuka, T. *Bioelectrochem. & Bioenerg.* **1996**, *41*, 201-203.

45. Pontié, M.; Bedioui, F.; Devynck, J. *Electroanalysis* **1999**, *11*, 845-850.