

Linköping University Post Print

Simultaneous use of electrochemistry and chemiluminescence to detect reactive oxygen species produced by human neutrophils

Sergey Shleev, Jonas Wetterö, Karl-Eric Magnusson and Tautgirdas Ruzgas

N.B.: When citing this work, cite the original article.

Original Publication:

Sergey Shleev, Jonas Wetterö, Karl-Eric Magnusson and Tautgirdas Ruzgas, Simultaneous use of electrochemistry and chemiluminescence to detect reactive oxygen species produced by human neutrophils, 2008, CELL BIOLOGY INTERNATIONAL, (32), 12, 1486-1496.

<http://dx.doi.org/10.1016/j.cellbi.2008.08.016>

Copyright: Elsevier Science B.V., Amsterdam

<http://www.elsevier.com/>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-16157>

Simultaneous use of electrochemistry and chemiluminescence to detect reactive oxygen species produced by human neutrophils

Sergey Shleev^{1,2,*}, Jonas Wetterö³, Karl-Eric Magnusson⁴, and Tautgirdas Ruzgas¹

¹ Biomedical Laboratory Science, Health and Society, Malmö University, 20506 Malmö, Sweden

² Laboratory of Chemical Enzymology, Institute of Biochemistry, 119071 Moscow, Russia

³ Rheumatology/AIR, Department of Clinical and Experimental Medicine, Linköping University, 58185 Linköping, Sweden

⁴ Medical Microbiology, Department of Clinical and Experimental Medicine, Linköping University, 58185 Linköping, Sweden

Abstract

A novel approach for the simultaneous optical and electrochemical detection of biologically produced reactive oxygen species has been developed and applied. The set-up consists of a luminol-dependent chemiluminescence assay combined with two amperometric biosensors sensitive to superoxide anion radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), respectively. The method permits direct, real time *in vitro* determination of both extra- and intracellular $O_2^{\bullet-}$ and H_2O_2 produced by human neutrophil granulocytes. The rate of $O_2^{\bullet-}$ production by stimulated neutrophils was calculated to about 10^{-17} mol s^{-1} per single cell. With inhibited NADPH oxidase, a distinct extracellular release of H_2O_2 instead of $O_2^{\bullet-}$ was obtained from stimulated neutrophils with the rate of about $3 \cdot 10^{-18}$ mol s^{-1} per single cell. When the H_2O_2 release was discontinued, fast H_2O_2 utilisation was observed. Direct interaction with and possibly attachment of neutrophils to redox protein-modified gold electrodes, resulted in a spontaneous respiratory burst in the population of cells closely associated to the electrode surface. Hence, further stimulation of human neutrophils with a potent receptor agonist (fMLF)

did not significantly increase the $O_2^{\bullet-}$ sensitive amperometric response. By contrast, the H_2O_2 sensitive biosensor, based on an HRP-modified graphite electrode, was able to reflect the bulk concentration of H_2O_2 , produced by stimulated neutrophils, and would be very useful in modestly equipped biomedical research laboratories. In summary, the system would also be appropriate for assessment of several other metabolites in different cell types, and tissues of varying complexity, with only minor electrode modifications.

Keywords: Superoxide anion radical; Hydrogen peroxide; Neutrophil; Biosensor, Luminol-dependent chemiluminescence, NADPH oxidase

* Corresponding author: Tel.: +46-40-665-7414; Fax: +46-40-665-8100.

E-mail address: sergey.shleev@mah.se

1. Introduction

Reactive oxygen species (ROS), *e.g.*, the superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), peroxy radical (ROO^{\bullet}), and hydrogen peroxide (H_2O_2), as well as their adducts with nitric oxide (*e.g.*, peroxynitrite, ONO_2^-), are essential in the human defence against infections. They also have important non-bactericidal functions in a number of cells and tissues, *viz.* in signal transduction, proliferation, thrombosis, inflammation, and cancer (Finkel, 1999; Lander, 1997; Stief, 2000). Human polymorphonuclear neutrophilic granulocytes (neutrophils) are the most abundant type of white blood cells and form an integral part of the human immune system. In circulation neutrophils are spheres with an average diameter and volume of 8 μm and 300 am^3 , respectively (Bainton, 1977; Simchowitz et al., 1993), whereas in peripheral blood smears their diameters increases up to 15-20 μm (*e.g.*, Bamberg and Johnson, 2002). Neutrophils respond to infection with a respiratory “burst” during which O_2 is reduced to $O_2^{\bullet-}$ by the NADPH oxidase. Superoxide may be released both to the phagosome and to the extracellular compartment (Babior, 1999). The short-lived ROS intermediate $O_2^{\bullet-}$ is potentially hostile to the host since it may exceed certain “antioxidant” levels locally in tissue (Finkel and Holbrook, 2000; White et al., 1994).

It is self-evident that reliable methods for ROS detection should have wide applicability. Such measurements are, however, seriously confounded by the evanescent nature of ROS and the multiple cellular mechanisms evolved to maintain these substances at low concentrations (Tarpey and Fridovich, 2001). Thus, techniques more particular than those presently available are required for the analysis of ROS. Ideally, a technique designed to measure cellular production of ROS should be very specific, sensitive, affordable, easy to handle and standardise, and also bio-inert (Dahlgren and Karlsson, 1999). No technique hitherto developed has satisfied all these criteria.

One popular concept for $O_2^{\bullet-}$ detection is based on the chemiluminescence (CL) principle (Tarpey and Fridovich, 2001). Several compounds have been used to enhance CL response, including luminol ($LumH_2$), isoluminol, lucigenin, and coelenterazine (Dahlgren and Karlsson, 1999; Faulkner and Fridovich, 1993; Lucas and Solano, 1992). $LumH_2$ is frequently used because it can access intracellular sites of $O_2^{\bullet-}$ generation, shows minimal toxicity and has a very high sensitivity (Faulkner and Fridovich, 1993; Yao et al., 2002). In aqueous alkaline solutions and in the presence of an oxidant, $LumH_2$ undergoes oxidation with emission of light (Roswell and White, 1978). H_2O_2 is the most useful oxidant, but it requires a catalyst, such as transition metal ions, hemin, or peroxidase. Instead of a catalyst, an electrode with positive applied potential can be used to produce electro-generated CL from the $LumH_2$ - H_2O_2 system (Epstein and Kuwana, 1967; Kuwana, 1963). Depending on the catalyst used, the pH range for efficient $LumH_2$ CL generally falls between 7 and 11 (Cormier and Prichard, 1968). Despite its popularity, $LumH_2$ is actually not ideally suited for specific $O_2^{\bullet-}$ detection within living cells for several reasons. Firstly, $O_2^{\bullet-}$ does not react directly with $LumH_2$ (Faulkner and Fridovich, 1993). Secondly, $LumH_2$ has an intrinsic ability to generate $O_2^{\bullet-}$, when its univalently oxidised form is autooxidised. In the case of activated neutrophils, univalent oxidants can be myeloperoxidase (MPO), or extracellular horseradish peroxidase (HRP) *in vitro*, plus H_2O_2 (Allred et al., 1980). Thirdly, the quantum yield of $O_2^{\bullet-}$ is limited at neutral (physiological) pH (Faulkner and Fridovich, 1993). Moreover, H_2O_2 is also able to trigger $LumH_2$ based luminescence (Lucas and Solano, 1992) and ONO_2^- , a product of the chemical reaction of $O_2^{\bullet-}$ and NO, reacts directly with $LumH_2$ to produce CL (Radi et al., 1993). Still, in the present study $LumH_2$ -dependent CL have been used as a very sensitive but not very selective method to detect total intra- and extracellular ROS production by neutrophils, taking into account the advantages and disadvantages of this compound.

Because ROS are very difficult to detect due to their high reactivity and short life-time (Amatore et al., 2000; Finkel and Holbrook, 2000; White et al., 1994), it is appealing to exploit

the advantages of electrochemical biosensors, *e.g.*, real-time detection with high sensitivity and selectivity. Although several electrochemical biosensors for the separate measurement of $O_2^{\bullet-}$ and H_2O_2 have been presented (Alpeeva et al., 2005; Campanella et al., 2000, 1997; Emreguel, 2005; Ferapontova et al., 2001; Ge and Lisdat, 2002; Lindgren et al., 2000; Lisdat et al., 1999; McNeil et al., 1995; McNeil and Manning, 2002; Scheller et al., 1999; Shleev et al., 2006), only a limited number of studies describe their simultaneous use (Krylov et al., 2006; Lvovich and Scheeline, 1997; Shipovskov et al., 2004). Furthermore, to our best knowledge simultaneous optical (using LumH₂) and electrochemical (using biosensors) monitoring of $O_2^{\bullet-}$ and H_2O_2 production by human neutrophil granulocytes has not been performed. Thus, the objective of the present work was to investigate ROS production in isolated human neutrophils using combined electrochemical and optical detection.

2. Materials and methods

2.1. Chemicals and buffers

Na_2HPO_4 , KH_2PO_4 , and NaCl were obtained from Merck GmbH (Darmstadt, Germany). Xanthine, fMLF-peptide (fMLF), diphenyleiodonium (DPI), LumH₂ (5-amino-2,3-dihydro-1,4-phtalazinedione), potassium superoxide (KO_2), and dimethylsulphoxid (DMSO) were from Sigma (St. Louis, MO, USA). 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) was from Pierce Biotechnology (Chester, UK). 11-mercapto-1-undecanol (MU) and 11-mercaptoundecanoic acid (MUA) were obtained from Aldrich (Steinheim, Germany). Absolute ethanol (99.7%) was from Solveco Chemicals AB (Täby, Sweden). All aqueous solutions were prepared using deionised water (18 M Ω) purified with a Milli-Q system (Millipore, Milford, CT, USA). The main buffers were phosphate-buffered saline (PBS; 10 mM sodium hydrogen phosphate, 10 mM potassium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.3), and Krebs-Ringer phosphate buffer supplemented with 10 mM glucose, 1.5 mM $MgSO_4$, and 1.1 mM $CaCl_2$ (pH 7.3; KRG). KRG was also prepared without the addition of $CaCl_2$ to avoid aggregation of neutrophils.

2.2. Proteins and enzymes

Pseudomonas aeruginosa azurin (MW 14.0 kDa), cytochrome *c* (cyt *c*, MW 12.4 kDa) from horse heart, and xanthine oxidase (XOD, Grade III, 1-2 units/mg, MW 300.0 kDa) from bovine milk were purchased from Sigma. Superoxide dismutase (SOD, 2500-7000 units/mg, MW 31.2 kDa) from bovine erythrocytes, horseradish peroxidase (HRP, Type VI, 300 units/mg, MW 40.0 kDa, RZ 2.7), and catalase (30000 units/mg, MW 250.0 kDa) from human erythrocytes were obtained from Boehringer Mannheim GmbH (Mannheim, Germany).

2.3. Cells

Peripheral human polymorphonuclear neutrophil granulocytes (neutrophils) were isolated from heparinised (10 U/ml) whole blood immediately following venipuncture of apparently healthy non-medicated volunteers (Böyum, A 1968; Ferrante, A, Thong, YH 1980). Whole blood was put on top of one part of Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) layered over four parts of Polymorphprep (Axis-Shield PoC AS) and centrifuged for 40 min at 480 x *g* at room temperature. The neutrophil fraction was collected and washed for 10 min at 480 x *g* in room temperature PBS, and erythrocytes were then eliminated by a short hypotonic lysis in ice-cold distilled water. Cells were then washed twice at 200 x *g* at 4°C in Ca²⁺-free KRG. The isolated neutrophils were counted in a Coulter Counter ZM Channelyser 256 (Coulter-Electronics Ltd., Luton, UK), showed excellent viability, <0.1 platelet contaminations per neutrophil, and were kept on melting ice until experiments were performed.

2.4. Preparation of the biosensors

2.4.1. Preparation of H₂O₂ sensitive HRP-modified graphite electrode

An HRP-modified spectrographic graphite electrode (HRP-SPGE) was used for electrochemical detection of hydrogen peroxide (Alpeeva et al., 2005; Ruzgas et al., 1996). Working electrodes with an outside diameter of 3.05 mm were prepared from rods of solid spectroscopic graphite type RW001 (Ringsdorff Werke GmbH, Bonn, Germany). The end of the graphite rod was polished on wet fine emery paper (Tufback Durite, P1200) and then carefully washed with deionised water. The enzyme was adsorbed onto the polished surface by placing 10 µl aliquots of a 2 mg/ml HRP solution on the dry electrode surface. Non-adsorbed enzyme was removed by repeated flushing in deionised water for at least 1 min. The HRP-SPGE electrode was then stored at room temperature for at

least 6 h in PBS for stabilisation before use. Calibration of the electrodes was performed using standard solutions of H_2O_2 , as well as superoxide production by reaction of KO_2 with water, as described in Shipovskov et al., 2004.

2.4.2. Preparation and electrochemical characterization of the $\text{O}_2^{\bullet-}$ sensitive gold electrodes

Three types of $\text{O}_2^{\bullet-}$ sensitive biosensors were prepared using two different redox proteins, azurin (Shleev et al., 2006) and cyt *c* (Ge and Lisdat, 2002; Manning et al., 1998), immobilised on thiol-modified gold disk electrodes from Bioanalytical Systems (model MF-2014, West Lafayette, IN, USA) with an area of about $2.5 \cdot 10^{-6} \text{ m}^2$. The bare gold electrode surface was first polished in DP-Suspension, and was then polished in alumina FF slurry (0.25 μm and 0.1 μm , respectively, Struers, Copenhagen, Denmark), rinsed in Millipore water with 10 min sonications in between. The electrodes were then cycled 30 times in 0.5 M H_2SO_4 , and kept in concentrated H_2SO_4 .

Prior to bio-modification gold electrodes were rinsed thoroughly with Millipore water. The first type of biosensor, based on azurin adsorbed on the DTSSP-modified gold electrode (azurin-DTSSP electrode), was prepared in accordance with our previous studies (Shleev et al., 2006). The second type of $\text{O}_2^{\bullet-}$ sensitive gold electrode (Cyt-DTSSP) was prepared according to Manning and co-authors (Manning et al., 1998). In the third case a gold electrode with cyt *c* adsorbed on a long-chain thiol layer (cyt-MU electrode) was used (Ge and Lisdat, 2002).

After preparation, the quality of the electrodes was controlled by cyclic voltammetry. Cyclic voltammograms (CVs) of the protein-modified electrodes were recorded using a one channel three-electrode potentiostat (BAS CV-50W Electrochemical Analyser with BAS CV-50W software v. 2.1, Bioanalytical Systems, West Lafayette, IN, USA) and one single-compartment 20 ml electrochemical cell. The reference electrode was an $\text{Hg} | \text{Hg}_2\text{Cl}_2 | \text{KCl}_{\text{sat}}$ (240 mV vs. NHE), and a platinum wire served as a counter electrode.

The calibration of the electrodes was performed using chemically or enzymatically produced superoxide as described elsewhere (Ge and Lisdat, 2002; Shleev et al., 2006; Tammeveski et al., 1998). Basic electrochemical characteristics of the biosensors used in the present study are listed in Table 1. The response time of the superoxide sensitive electrodes is less than 1 ms, as briefly described below. Thus, it is in principle possible to perform real-time monitoring of superoxide radicals using all three types of biosensors described.

The approximate response time (3τ), which is related to recharging of the electrode, can be calculated from Eq. 1 (Bard and Faulkner, 1980).

$$3\tau = R_{\text{sol}} \times C_d \quad (\text{Eq. 1})$$

where R_{sol} is the resistance of the solution, *ca.* 200 Ω in our studies, and C_d is the double layer capacitance of a protein monolayer based electrode, *ca.* $10^{-5} \text{ F cm}^{-2}$.

Table 1. Basic electrochemical characteristics of the biosensors used in present study

Type of biosensor	Analyte	Sensitivity (A m ⁻² M ⁻¹)	Stability (h)	Preparation time (h)	Reference
HRP-SPGE	H ₂ O ₂	5.0·10 ²	> 8	≈ 4	(Alpeeva et al., 2005; Ruzgas et al., 1996)
Azurin-DTSSP	O ₂ ^{•-}	6.0·10 ²	≈ 5	≈ 20	(Shleev et al., 2006)
Cyt-DTSSP	O ₂ ^{•-}	0.5·10 ²	6-8	≈ 20	(Tammeveski et al., 1998)
Cyt-MU	O ₂ ^{•-}	2.5·10 ²	≥ 8	≈ 30	(Ge and Lisdat, 2002)

An approximate response time (3τ), which is related the diffusion of superoxide to the electrode surface, can be calculated from Eq. 2.

$$3\tau = \frac{\delta^2}{4D} \quad (\text{Eq. 2})$$

where δ is the distance between the electrode surface and superoxide (1 micron in our studies) and D is the diffusion coefficient of superoxide ions in the solution ($2 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$).

2.5. Detection of ROS

2.5.1. Luminometric detection of ROS (LumH₂-dependent CL)

The intra- and extracellular neutrophil generation of ROS was studied by LumH₂-dependent CL in a six-channel Biolumat (LB 9505 C, Berthold Co., Wildbaden, Germany). Measurements were performed in polypropylene tubes with neutrophils ($1 \cdot 10^6$ up to $5 \cdot 10^6$ cells/ml) at 37°C in KRG with or without HRP (4 U/ml) and LumH₂ (56.4 μM). Intracellular ROS were also detected in the presence of scavengers, SOD (200 U/ml) and catalase (2000 U/ml), as well as an inhibitor of the NADPH oxidase, DPI (5.0 μM). In some experiments neutrophils were given 5 min for pre-warming and stabilisation before being stimulated with 0.1 μM fMLF.

2.5.2. Electrochemical detection of O₂^{•-} and H₂O₂

The extracellular generation of O₂^{•-} and H₂O₂ in isolated neutrophils was studied using either a multi-channel custom-built potentiostat (Institute of Biochemistry, Vilnius, Lithuania; http://www.tpa.lt/5FP/LIFE/life_intellisens.html), or a one channel CV-50W potentiostat. Initially, two working electrodes, O₂^{•-} and H₂O₂ sensitive biosensors, were used simultaneously in one Biolumat channel. Different potentials were applied to the HRP-SPGE and O₂^{•-} sensitive gold working electrodes, -50 mV and +250 mV,

respectively, and thus two chronoamperometric curves were obtained simultaneously. Finally, only one biosensor was used, either a $O_2^{\bullet-}$ or a H_2O_2 sensitive electrode, as described below. *In vitro* electrochemical measurements were performed using a silver wire in PBS as a combined reference and counter electrode. All measurements described were performed at least in triplicate using freshly prepared $O_2^{\bullet-}$ and H_2O_2 sensitive electrodes.

3. Results

The H_2O_2 and $\text{O}_2^{\bullet-}$ sensitive biosensors were combined with the LumH₂ CL assay to estimate the ROS production by human neutrophils. It was found that simultaneous use of both H_2O_2 and $\text{O}_2^{\bullet-}$ sensitive electrodes in one Biolumat channel with accompanying CL detection resulted in significant interference between the electrochemical and optical detections, as well as in minor cross talk between two electrochemical signals. For example, LumH₂ induced a substantial background current from the HRP-SPGE because of enzymatic and, possibly, electrochemical reactions of LumH₂ on the electrode surface. Thus, to avoid interferences and cross talks, electrochemical and optical monitoring were performed in separate Biolumat channels and only one biosensor, either a H_2O_2 or a $\text{O}_2^{\bullet-}$ sensitive electrode, was used for each experimental run (Fig. 1). In order to normalize the results, the electrochemical and spectral measurements were started simultaneously along with the subsequent addition of cells and chemical compounds.

3.1. Chemiluminescence detection of ROS in combination with the H_2O_2 sensitive biosensor

First, the HRP-SPGE and LumH₂ were combined. Initially, both electrochemical and CL signals were stabilised and baselines were established. After that, aliquots carrying $1 \cdot 10^6$ up to $5 \cdot 10^6$ neutrophils per ml were added to the electrochemical and optical compartments (Fig. 1) and distinct signals were obtained. A typical CL signal and a current response recorded with the Biolumat and HRP-SPGE are shown in Fig. 2A and B, respectively. One part of the immediate response upon addition of neutrophils (as indicated by arrows in all figures) shown by the CL assay can be explained by the neutrophil transfer to the 37°C milieu after storage on melting ice (Fig. 2A). For comparison, modest mechanical disturbance of the electrochemical system, and possibly cells attaching to the electrode surface, also contributed significantly to the huge current

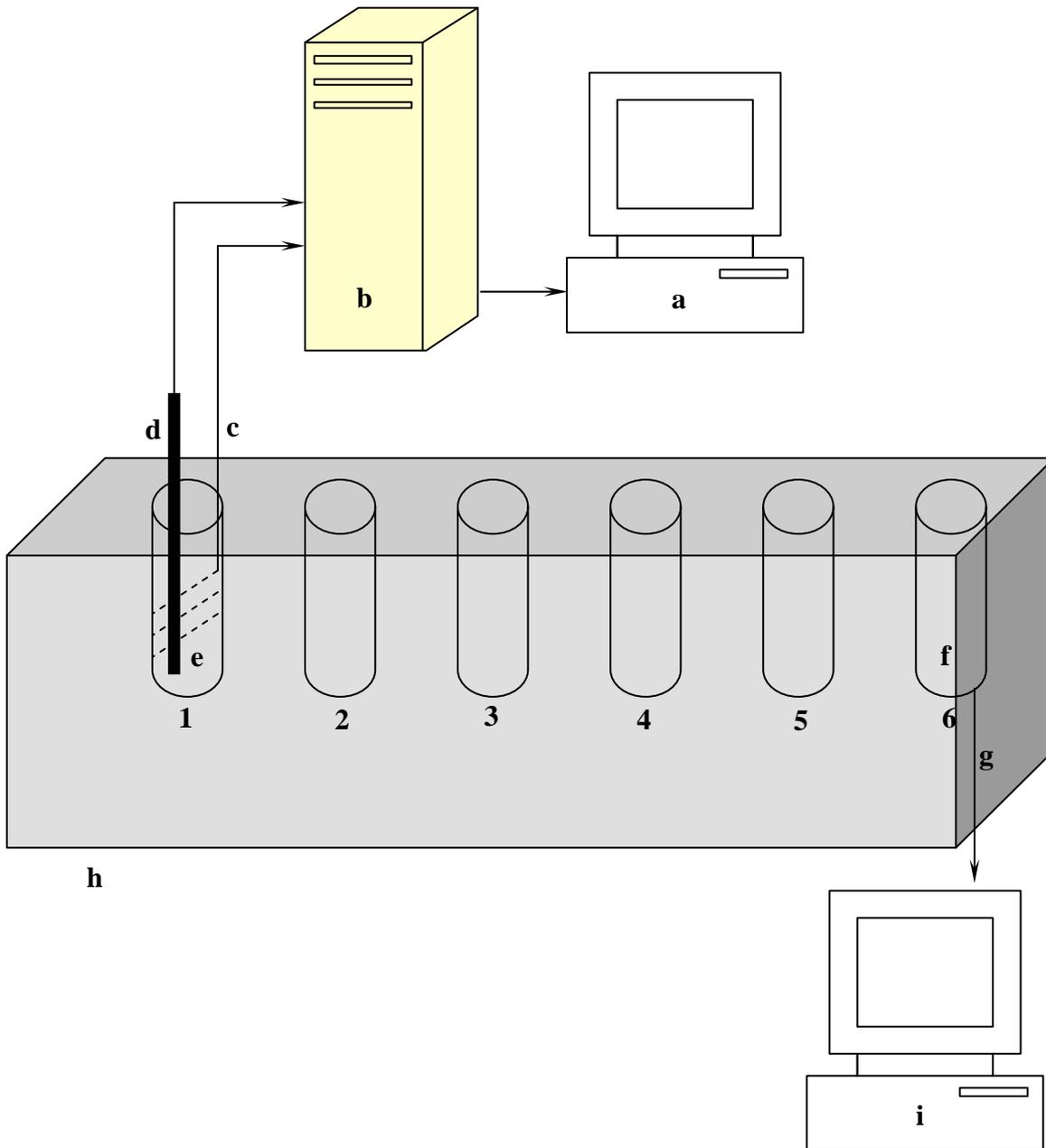


Figure 1. Detection scheme of the optical-electrochemical set-up to monitor ROS production by human neutrophils. a – computer for the detection of $O_2^{\bullet-}$ or H_2O_2 signals; b – potentiostat CV-50W; c – combined reference and counter electrode (Ag wire); d – working electrode ($O_2^{\bullet-}$ -sensitive gold electrode or H_2O_2 -sensitive graphite electrode); e – electrochemical cell (first channel of Biolumat); f – optical compartment (channel No. 6 of Biolumat); g – optical signal from the LumH₂-dependent CL compartment; h –channel No. 6 of Biolumat; i – computer for the detection of CL, 1-6 – corresponding channels of the Biolumat.

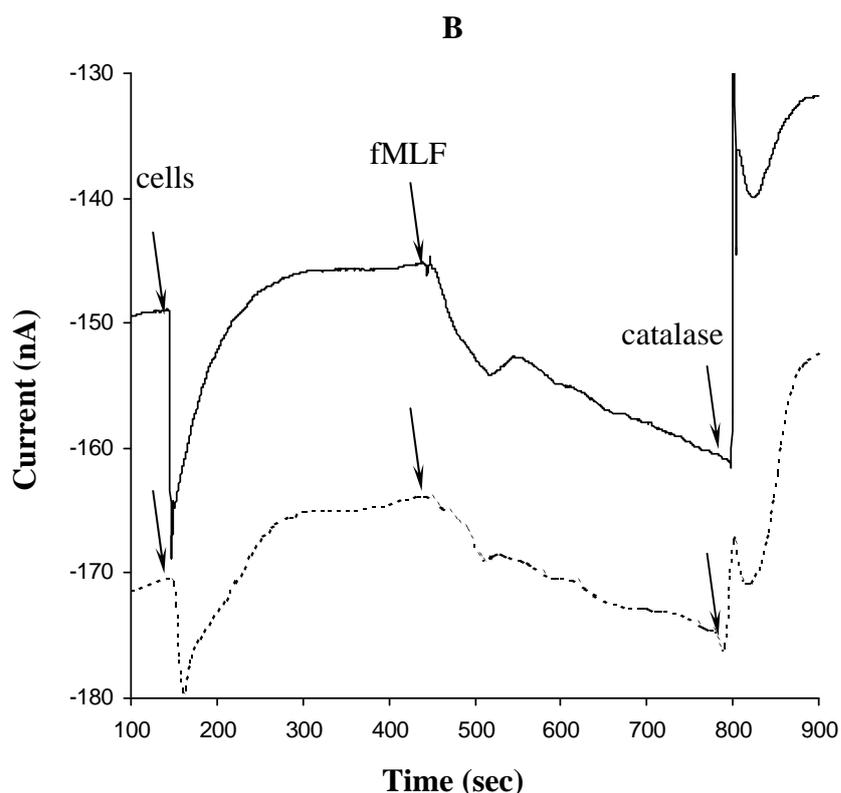
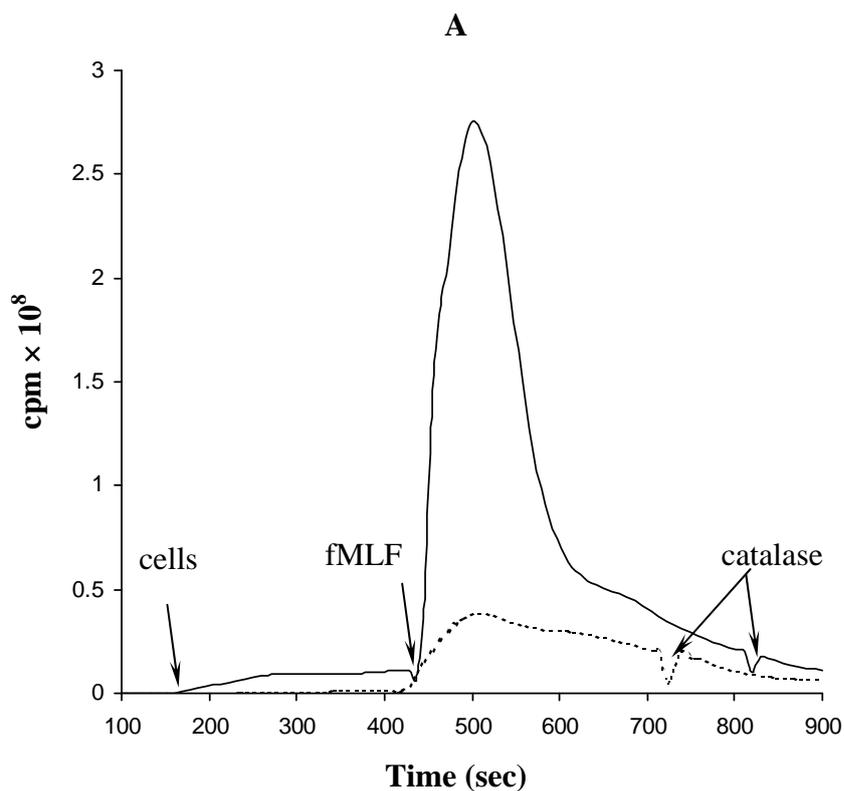


Figure 2. Response curves for ROS from neutrophils following stimulation with $0.1 \mu\text{M}$ fMLF in the presence (solid line) and absence (dotted line) of 4 U/ml extracellular HRP.

(A) LumH₂-dependent CL signal (KRG buffer; $56.4 \mu\text{M}$ LumH₂).

(B) Amperometric detection of extracellular H₂O₂ using the HRP-modified SPGE (KRG buffer). Arrows indicate the addition of neutrophils, fMLF, and catalase into cuvettes equipped with the HRP-modified SPGE-based biosensor or containing the luminol reagent. Final concentration of cells in the cuvette: 10^6 cells/ml; 37°C ; Cpm = counts per minute.

step obtained directly after addition of cells (Fig. 2B). However, a minor contribution to the current step corresponding to ROS production could depend on the long stabilisation time, and/or the reductive direction of the current compared to the oxidative one that is always obtained with $O_2^{\bullet-}$ sensitive biosensors (Fig. 2B). While a low steady-state level of the CL signal could be seen after stabilisation (corresponding to $3 \cdot 10^6$ - $9 \cdot 10^6$ counts per min), the electrochemical response was just at the background level. The data scattering in the CL assay is probably the result of different durations between neutrophil isolation and the ROS measurements.

For the following 300 s, the cells warm up and the signals from both methods stabilise (Fig. 2). Addition of a potent receptor agonist (fMLF) resulted in a marked CL signal, which reached its maximum at about 500 s, *i.e.* approximately 100 s after stimulation. The peak response was *ca.* 30 times higher ($2.4 \cdot 10^8$ - $5.5 \cdot 10^8$ cpm), when compared to the signal without stimuli (Fig. 2A). While the CL signal decreased rapidly after it had reached maximum, the accumulation of H_2O_2 could still be observed, however, with a significantly reduced rate of accumulation (*cf.* Figs. 2A and 2B). The extracellular concentration of H_2O_2 at 507 s, *i.e.*, at the maximum CL, coincides perfectly with a small peak on the chronoamperometric curve. Here the H_2O_2 concentration was calculated to be close to 0.6 μM (Fig. 2). H_2O_2 continued to accumulate and the highest level of extracellular H_2O_2 produced by fMLF-stimulated neutrophils was found to be *ca.* 3.0 μM . Addition of catalase, a specific H_2O_2 utilising enzyme catalysing peroxide disproportionation, resulted in the complete loss of the electrochemical signal from the H_2O_2 -SPGE. Indeed, it rapidly reached the background level, confirming the selectivity of the biosensor. Both the CL signals and the extracellular concentration of H_2O_2 were dependent on the concentration of neutrophils from $1 \cdot 10^6$ to $5 \cdot 10^6$ cells per ml.

It is interesting to compare the results from two independent methods, electrochemistry and CL, in the presence and absence of the extracellular catalyst HRP. The data (Fig. 2) displays a drop in both the electrochemical and CL signal in the absence of the enzyme with similar

temporal patterns of both curves (*cf.* solid and dotted lines in Fig. 2). In the absence of HRP the CL signal decreased to 1/7 (*i.e.* by 86%) of its value in the presence of the enzyme, whereas the total electrochemical signal decreased by only 25%. A decrease in the CL signal without extracellular HRP could be expected since peroxidases are typical catalysts required for the light emission reactions at neutral pH (Cormier and Prichard, 1968).

Inhibition of the NADPH oxidase by DPI resulted in dramatic changes in both the patterns and amplitudes of the optical and electrochemical signals (Fig. 3). Firstly, both signals dropped to near background levels immediately following DPI addition. Secondly, the fMLF-stimulated responses from Biolumat and the H₂O₂ sensitive biosensor were approximately 70 and 2 times lower than without inhibition of the enzyme after cell stimulation. Incidentally, the rate of H₂O₂ accumulation during the initial inhibition stage decreased by 400%, *e.g.* from 0.12 nA s⁻¹ for intact cells to 0.03 nA s⁻¹ for DPI-inhibited neutrophils. Thirdly, an additional fMLF-triggered secondary broad peak (Fig 3A, at 950s) appeared in the CL response, while no further accumulation of extracellular H₂O₂ was observed in the electrochemical system. In contrast to the experiments with intact neutrophils, where further accumulation of H₂O₂ was observed, no H₂O₂ was detected in the solution at 500 s after the stimulation in the presence of DPI (Fig. 3B). Finally, the peaks of the optical and electrochemical curves did not coincide in time after the inhibition of NADPH oxidase contrary to findings for intact neutrophils (*cf.* Fig. 3A and B).

3.2. Chemiluminescence detection of ROS in combination with O₂^{•-} sensitive biosensors

The basic parameters of the azurin-DTSSP, cyt-MU, and cyt-DTSSP modified electrodes are listed in Table 1. The sensitivity of the biosensors towards O₂^{•-} were approximately 6.0·10², 2.5·10², and 0.5·10² A m⁻² M⁻¹, respectively. Thus, macroscale electrodes would generally be able to sense the typically very small extracellular amount of O₂^{•-}, which may be released *in vivo*. To compare the different assays, all experiments were synchronised as far as possible.

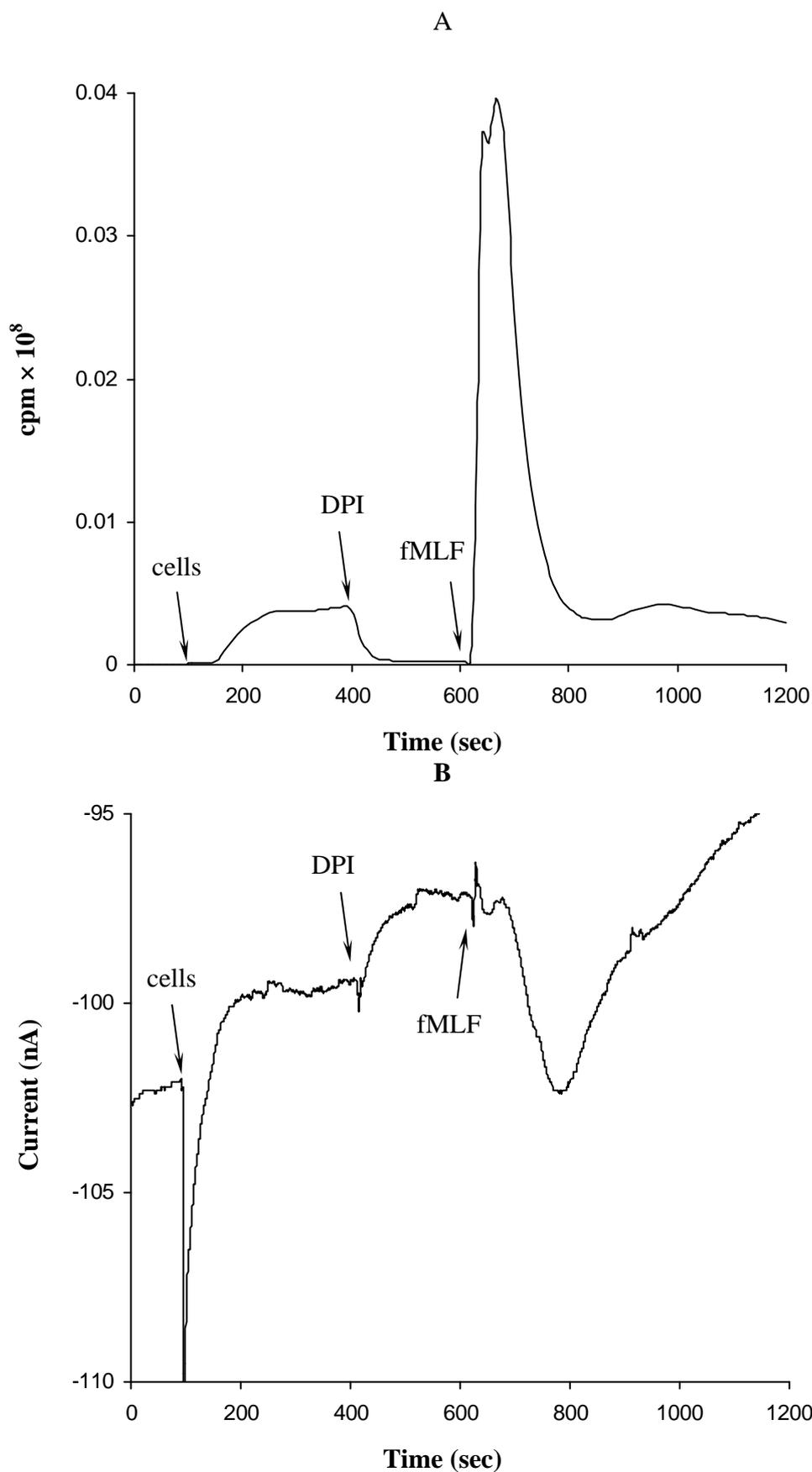


Figure 3. Response curves for ROS from neutrophils following stimulation with $0.1 \mu\text{M}$ fMLF after the addition of $5 \mu\text{M}$ DPI.

(A) LumH₂-dependent CL signal (KRG buffer; $56.4 \mu\text{M}$ LumH₂; 4 U/ml HRP).

(B) Amperometric detection of extracellular H₂O₂ using the HRP-modified SPGE (KRG buffer; 4 U/ml HRP).

Arrows indicate the addition of neutrophils, DPI, and fMLF into cuvettes equipped with the HRP-modified SPGE-based biosensor or containing the luminol reagent. Final concentration of cells in the cuvette: 10^6 cells/ml; 37°C .

Acknowledging the complexity of studies on radicals in biological systems, the use of several alternative sensing chemistries is important to avoid misinterpretation due to possible artefacts. It should be emphasised also that the three different $O_2^{\bullet-}$ sensitive biosensors showed qualitatively similar results (not shown). The azurin-DTSSP-modified gold electrode is probably the most sensitive biosensor for $O_2^{\bullet-}$ detection reported so far, and hence, only the data obtained with this particular electrode are presented and discussed in detail below.

From the beginning of the measurements both electrochemical ($O_2^{\bullet-}$ sensitive biosensors) and optical (ROS sensitive CL assay) signals were stabilised. After the stabilisation the neutrophils ($1\text{-}5\cdot 10^6$ cells per ml) were added to the electrochemical and CL compartments. Typical azurin-DTSSP electrode current and CL responses are shown in Fig. 4. The data from the CL assay was similar to the previous experiments (compare Fig. 2A with Fig. 4A and B). The addition of catalase to the luminometer compartment resulted in a significant drop of the CL signal (Fig. 4B).

Contrary to the H_2O_2 sensitive electrode, the azurin-based biosensors displayed pronounced current responses (0.5 - 2.0 nA), corresponding to an extracellular $O_2^{\bullet-}$ concentration of about 0.5 - 2.0 μM , prior to the stimulation of the neutrophils (Figs. 4C, 6B). After 300 s of equilibration the signals from either assay were stable and fMLF was added simultaneously to both channels. Surprisingly, the agonist increased the biosensor response only moderately, *i.e.*, by 0.1 nA (corresponding to *ca.* 0.1 μM of $O_2^{\bullet-}$) in the case of the azurin-DTSSP electrode (Fig. 4D). Subsequent addition of catalase resulted in a sharp decrease of the CL signal (Fig. 4B), and a very small amperometric increase, respectively (Fig. 4D).

In another experiment, HRP was present in the electrochemical cell (Fig. 5B), but this did not affect the electrochemical signal. By contrast, addition of SOD instead of catalase resulted in a sharp decrease of both the electrochemical (~ 0.75 nA, Fig. 5B) and the optical ($\sim 1\cdot 10^8$ cpm, Fig. 5A) signal. However, here the current from the azurin-DTSSP electrode did not reach the

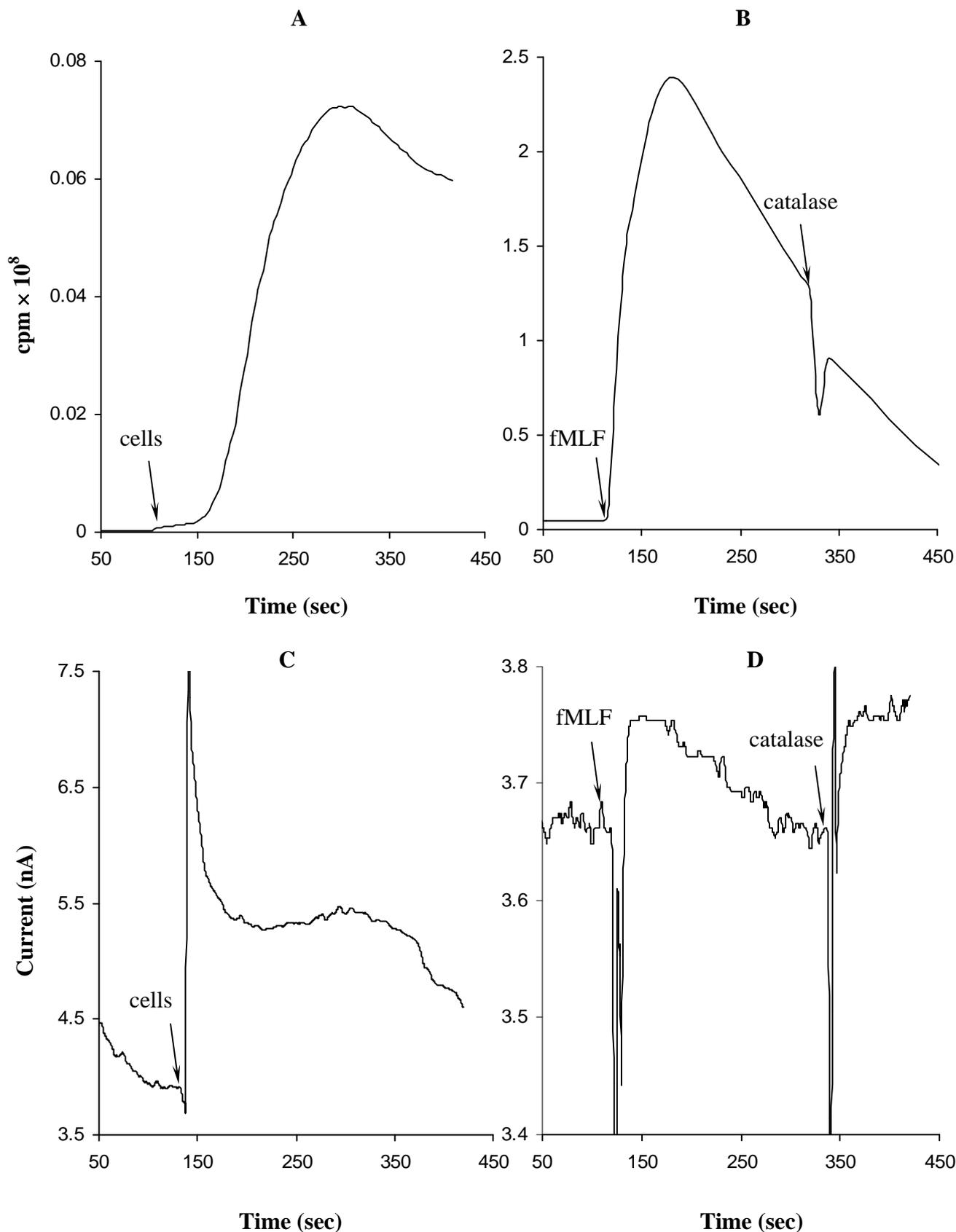


Figure 4. Response curves for ROS from neutrophils following stimulation with $0.1 \mu\text{M}$ fMLF. (A and B) LumH₂-dependent CL signal (KRG buffer; $56.4 \mu\text{M}$ LumH₂; 4 U/ml HRP). (C and D) Amperometric detection of extracellular O₂[•] using the Azurin-DTSSP-immobilised gold electrode (KRG buffer only). Arrows indicate the addition of neutrophils, fMLF, and catalase into cuvettes equipped with the azurin-DTSSP-based biosensor or containing the luminol reagent. Final concentration of cells in the cuvette: 10^6 cells/ml; 37 °C.

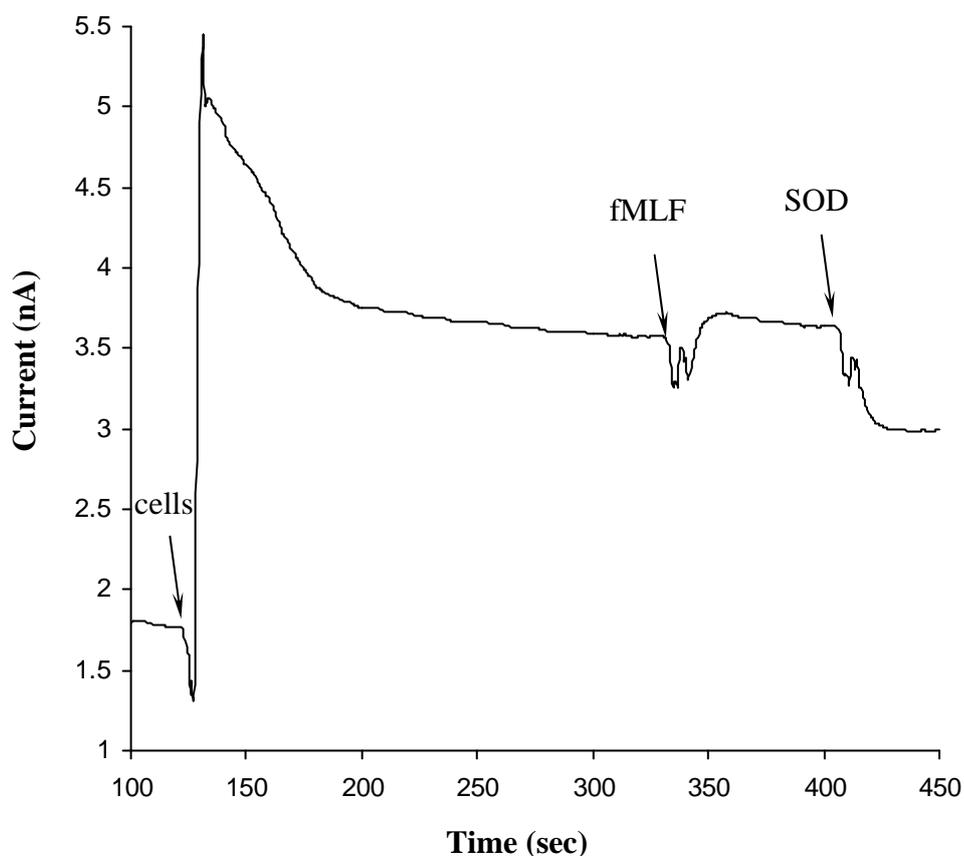
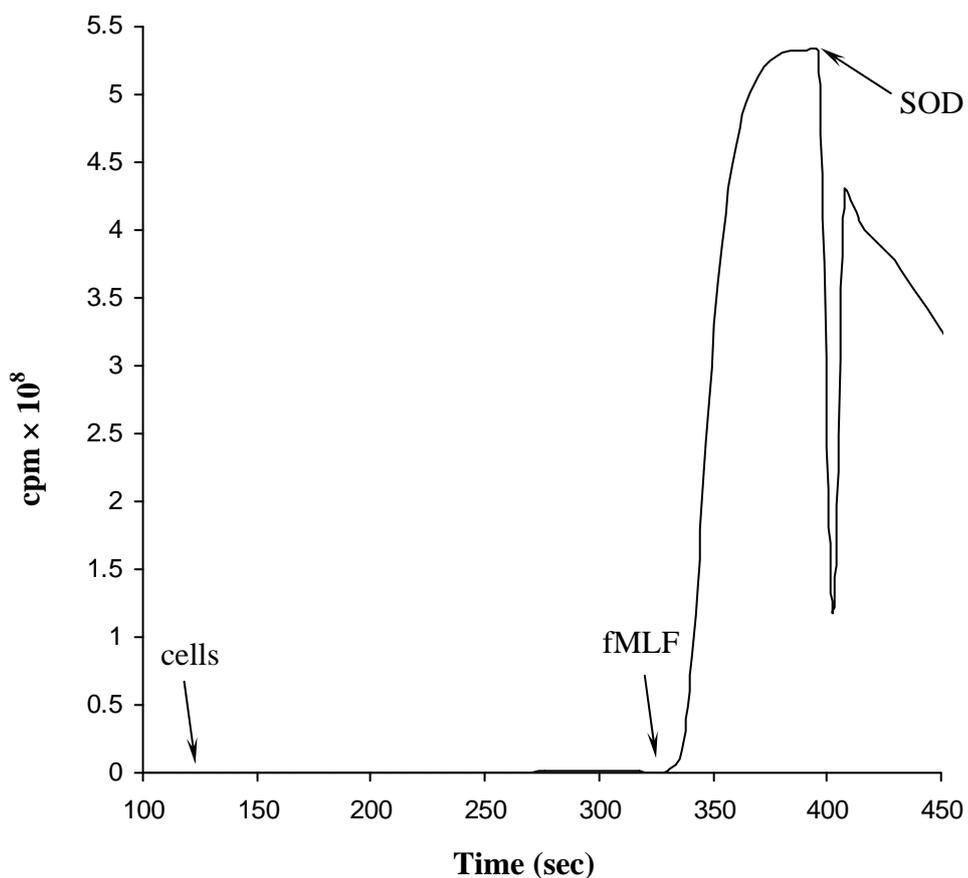


Figure 5. Response curves for ROS from neutrophils following stimulation with $0.1 \mu\text{M}$ fMLF.

(A) LumH₂-dependent CL signal (KRG buffer; $56.4 \mu\text{M}$ LumH₂; 4 U/ml HRP).

(B) Amperometric detection of extracellular O₂* using the azurin-DTSSP-immobilised gold electrode (KRG buffer; 4 U/ml HRP).

Arrows indicate the addition of neutrophils, fMLF, and SOD into cuvettes equipped with the azurin-DTSSP-based biosensor or containing the luminol reagent. Final concentration of cells in the cuvette: 10^6 cells/ml; 37 °C.

background level, which is contrary to what we observed with the HRP-based H₂O₂ sensitive biosensor after the addition of catalase (*cf.* Figs. 2B and 5B).

The step of the current from the O₂^{•-} sensitive biosensors directly after neutrophil addition, the increase in the reductive current after fMLF stimulation, and the SOD depleted current, were all highly depended on the amount of neutrophils in the solution, ranging from 1·10⁶ to 3·10⁶ cells per ml. A further increase of the cell density did not affect the O₂^{•-} sensitive electrochemical signals (data not shown).

To clarify the nature of the initial current step after cell injection, additional experiments were performed with unstimulated neutrophils (Fig. 6). The cell transfer from 4°C to 37°C resulted in an altered CL signal (Fig. 6A) suggesting a mainly temperature dependent production of ROS. Inhibition of neutrophils by DPI caused an almost complete disappearance of both the optical and electrochemical signals, confirming the cellular origin of the ROS (Fig. 6).

4. Discussion

Both the LumH₂-dependent CL signal and the current from the ROS sensitive biosensors depended quantitatively on the cell density and addition of different scavengers, *i.e.* SOD and catalase, which is in good agreement with previously published data (Allred et al., 1980; Dahlgren et al., 1985; Dahlgren and Karlsson, 1999; McNei et al., 1989, 1992, 1995). To evaluate the complex experiments several aspects have to be considered. Firstly, the LumH₂-dependent CL assay reflects both intra- and extracellular production of ROS, whereas the electrochemical signal corresponds specifically to the O₂^{•-} and H₂O₂ released from neutrophils. When using HRP as a catalyst, O₂^{•-} is the primary extracellular oxygen species in the peroxidase-catalysed excitation of LumH₂, and thus H₂O₂ released from the cells does not significantly affect the CL (Dahlgren and Karlsson, 1999; Lock et al., 1988; Lundqvist and Dahlgren, 1996). Secondly, SOD and catalase can only scavenge extracellular metabolites. However, these scavengers might still affect intracellular responses since the removal of ROS from the medium,

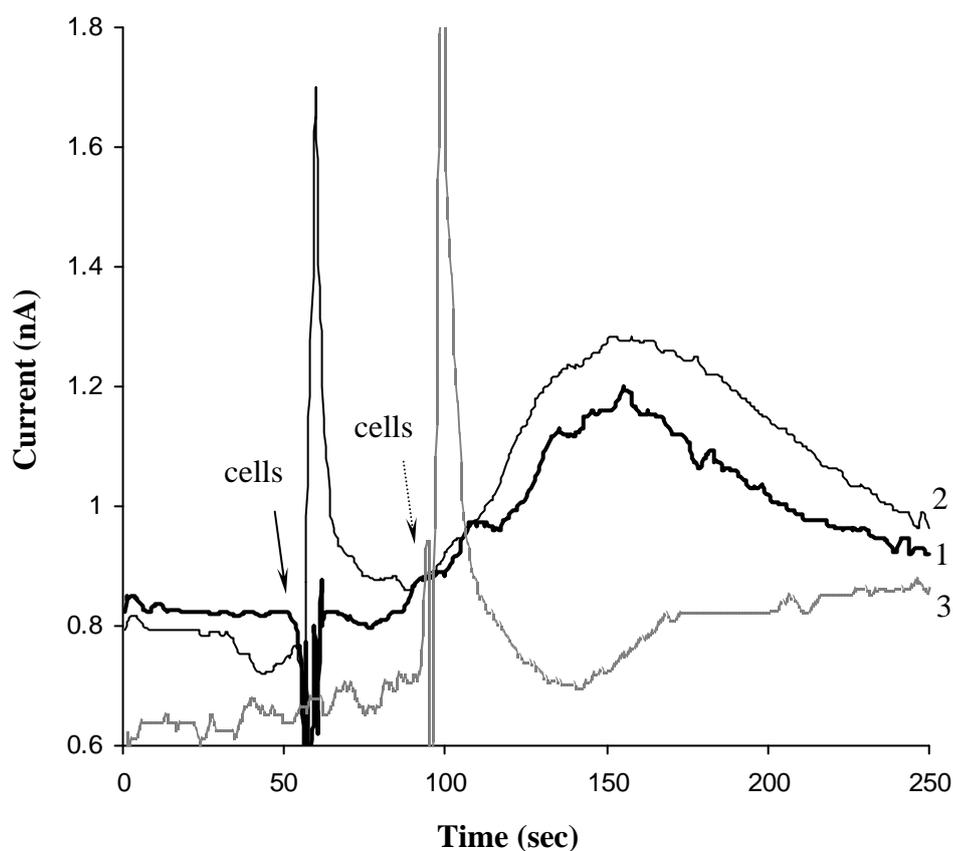
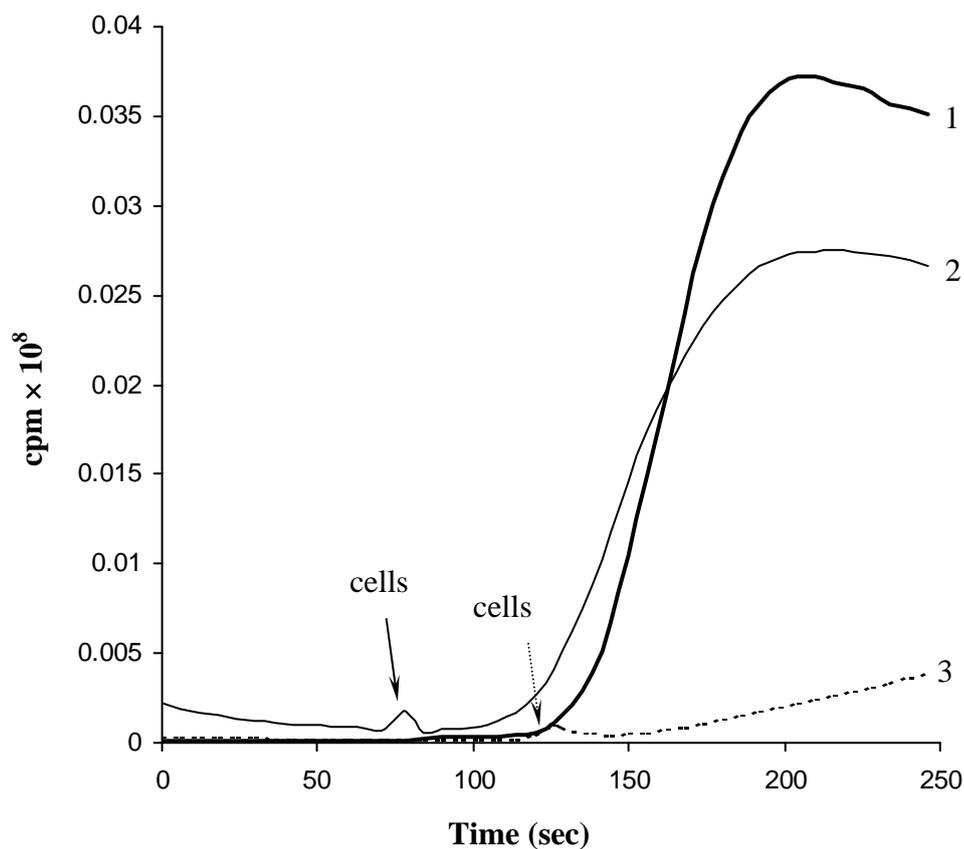


Figure 6. Response curves for ROS from unstimulated neutrophils.

(A) LumH₂-dependent CL signal (KRG buffer; 56.4 μM LumH₂; 4 U/ml HRP).

(B) Amperometric detection of extracellular O₂^{*} using the Azurin-DTSSP-immobilised gold electrode (KRG buffer; 4 U/ml HRP).

1, bold lines – cells from melting ice directly; 2, solid lines – cells pre-incubated for 5 min at 37 °C; 3, dotted lines – cells pre-incubated for 5 min at 37 °C with 5 μM DPI.

Arrows indicate the addition of neutrophils into cuvettes equipped with the azurin-DTSSP-gold biosensor or containing the luminol reagent. Final concentration of cells in the cuvette: 10⁶ cells/ml; 37 °C.

could promote the expulsion of H_2O_2 and $\text{O}_2^{\bullet-}$ from the cytoplasm to maintain equilibrium (Halliwell and Gutteridge, 1990). Thirdly, only $\text{O}_2^{\bullet-}$ in close proximity to the electrode contributes to the amperometric response due to the fast spontaneous dismutation to H_2O_2 at neutral pH. A rough calculation based on the diffusion coefficient of O_2 in water ($1.8 \cdot 10^{-9} \text{ m}^2 \text{ s}^{-1}$) and the short life time of $\text{O}_2^{\bullet-}$ (less than 0.1 s at physiological conditions) indicates that the critical distance does not exceed 15 μm . Coincidentally, the extracellular H_2O_2 concentration can be precisely measured *via* electrochemical detection in bulk solution since it is quite stable compared to $\text{O}_2^{\bullet-}$.

Taking all these facts together the following conclusions can be made, and a simple mathematical treatment of the data can be performed. Firstly, since there was no significant effect of LumH₂ itself on neutrophils in our studies, on inhibition of $\text{O}_2^{\bullet-}$ release (Faldt et al., 1999) or on LumH₂-induced CL (Allred et al., 1980), in the absence of extracellular HRP the LumH₂-dependent CL signal corresponds to a first approximation to intracellular production of ROS. Therefore, the difference between the two curves presented in Fig. 2A reflects the extracellular release of $\text{O}_2^{\bullet-}$ from neutrophils. Moreover, the maximum of the LumH₂-dependent CL signal almost coincided with the first maximum on the chronoamperometric curve from the H_2O_2 sensitive biosensor (*cf.* Fig. 2A and B). In accordance with chemical stoichiometry, one mole of H_2O_2 is produced from 2 moles of $\text{O}_2^{\bullet-}$ through fast spontaneous dismutation. After fMLF stimulation a respiratory “burst” of human neutrophils results in a very rapid, but rather short-lived (~ 100 s duration) extracellular release of $\text{O}_2^{\bullet-}$, $\sim 10^{-17} \text{ mol s}^{-1}$ from a single neutrophil, as calculated from the maximal concentration of $\text{O}_2^{\bullet-}$, 1.2 μM from 0.6 μM H_2O_2 ; Fig. 2B, produced in 100 s by 10^6 cells in 1 ml volume. Thus, in one second a single cell releases $\sim 10^7$ molecules of $\text{O}_2^{\bullet-}$ upon stimulation. This is a quite reasonable value taking into account the reported turnover number of NADPH oxidase in human neutrophils of 12-25 s^{-1} (Glass et al., 1986).

Secondly, inhibiting NADPH oxidase with DPI drastically decreased both intra- and extracellular ROS production from either fMLF-stimulated or temperature-stressed neutrophils (Fig. 3). In fact, the total CL signal, as calculated from the total area under the graph, decreased by more than a factor of 150 compared to the signal from unimpaired cells. In contrast, the total amount of H₂O₂, as determined from the total area of the electrochemical signal, produced by the same number of cells was found to be only 10 times smaller than without inhibition. Moreover, while the maximum value of the optical signal was found to be 70 times lower upon addition of DPI, (*cf.* Fig. 2A and 3A), the corresponding peaks on the chronoamperometric curves differed only by a factor of two (*cf.* Fig. 2B and 3B). It is obvious that this discrepancy of more than one order of magnitude between the electrochemical and optical data cannot be explained by a difference in extra- and intracellular production of ROS. The experiments showed, however, a substantial extracellular release of H₂O₂ instead of O₂^{•-} from neutrophils with DPI-inhibited NADPH oxidase; in the initial phase it was $\sim 0.3 \cdot 10^{-17} \text{ mole s}^{-1}$ of H₂O₂ by a single cell, based on the H₂O₂ concentration of $\sim 0.4 \text{ }\mu\text{M}$ produced in 150 s by 10⁶ neutrophils in 1 ml. Thus, our observations confirm a satisfactory selectivity of the LumH₂-dependent CL assay towards extracellular O₂^{•-} in the presence of HRP. Given the stoichiometry of the O₂^{•-} dismutation reaction in non-inhibited cells releasing O₂^{•-}, the extracellular production of H₂O₂ by stimulated neutrophils with inhibited NADPH oxidase is halved as compared to the usual respiratory “burst”. Thus, in the presence of DPI, neutrophils still respond to fMLF, but by releasing H₂O₂ instead of O₂^{•-}. Previously published data support H₂O₂ generation by neutrophils with inhibited NADPH oxidase, when stimulated with 6-formylpterin (Yamashita et al., 2001). This bifurcating behaviour of neutrophils may be important physiologically, and could possibly be exploited for medical applications.

As mentioned earlier, pre-incubation of cells at 37°C before injection into the Biolumat channels resulted in a significant drop in the CL signal, reflecting the production of ROS by stressed neutrophils during warming after storage on melting ice. The rate and total amount of

ROS produced by the stressed neutrophils were, however, almost negligible compared to those of stimulated cells (Figs. 2A and 6A). At the same time the $O_2^{\bullet-}$ sensitive electrochemical signals seemed insensitive to pre-warming, whereas DPI inhibition resulted in an almost complete disappearance of both optical and electrochemical signals, confirming their $O_2^{\bullet-}$ origin (Fig. 6). The current from the $O_2^{\bullet-}$ sensitive biosensor corresponds specifically to $O_2^{\bullet-}$ being released from neutrophils in very close proximity to the electrodes, *i.e.* neutrophils directly adhering to the biosensors. This proposition also helps us explain the unexpected results when $O_2^{\bullet-}$ sensitive electrodes were used. Hence, while the dominant population of neutrophils, *i.e.* non-adsorbed, react as expected on the specific fMLF-stimulation by a respiratory “burst”, the cells on the electrode surface are already in some sense stimulated by the electrode surface or the adsorbed redox proteins, thus giving a seemingly insignificant response to fMLF (Figs. 4 and 5). Moreover, SOD inhibited the electrochemical signal significantly, but not back to the background level, possibly due to steric factors prohibiting the enzyme to penetrate between the cells and the electrode (Fig. 5B). On the other hand, addition of catalase increased the signal (Fig. 4D), most probably by removal of H_2O_2 from the bulk medium and promoting the generation of $O_2^{\bullet-}$ in the cytoplasm. Yet, the current increase was limited by the fixed number of neutrophils on the electrode surface. Using the area of the gold electrodes ($2.5 \cdot 10^{-6} \text{ m}^2$) and the size of a single neutrophil (0.2 nm^2), the approximate number of cells attached to the electrode surface was calculated to be $1.25 \cdot 10^4$. Taking into account the known life time of $O_2^{\bullet-}$ (*ca.* 0.1 s), the rate of $O_2^{\bullet-}$ production by stimulated neutrophils ($10^{-17} \text{ mole s}^{-1}$ of $O_2^{\bullet-}$ per single neutrophil), and the small confined volume ($\approx 10^{-11} \text{ m}^3$) limited by several hundred nm distance between the electrode and cells, the local steady-state concentration of $O_2^{\bullet-}$, which arises from the equilibrium between $O_2^{\bullet-}$ production and spontaneous dismutation, can be estimated to reach several μM in a few hundred sec after injection of neutrophils. Interestingly, closely related values for the steady-state concentration of $O_2^{\bullet-}$ were indeed obtained from the $O_2^{\bullet-}$ sensitive biosensors, *i.e.* about $1 \mu\text{M}$. Electrochemical detection of $O_2^{\bullet-}$ from living cells in bulk solution

is accordingly only possible in a very small volume, where cells are separated somehow from the electrode surface. This approach has recently been realised by Chang and co-workers (2005a, 2005b), where cells were attached to a glass spacer or a COSTAR[®] membrane insert, a few hundred μm away from the surface of an $\text{O}_2^{\bullet-}$ sensitive biosensor. An excellent correlation between the rate of the current increase from $\text{O}_2^{\bullet-}$ sensitive gold electrodes and the number of cells seeded was obtained (Chang et al., 2005a, 2005b), whereas in the present work, and some other studies (McNeil et al., 1992, 1995), the response curve was not linear, but showed a saturation behaviour at higher neutrophil loadings, reflecting the attachment of the latter to the electrode surface.

To meet all the criteria for sensitive and selective measurements of ROS produced by living cells, the design of an electrochemical system should be recognised as the most promising device for real time determination of steady-state $\text{O}_2^{\bullet-}$ concentrations without interference from cellular metabolism and associated regulatory pathways (Campanella et al., 2000; Chang et al., 2005a, 2005b; Manning et al., 1998; McNeil and Manning, 2002). However, placing the biosensors in a compartment with a spectral system (Chang et al., 2005b) requiring a dye probe to produce the optical signal, limits the applicability. Therefore, we performed the electrochemical and optical assays in two separate compartments along with simultaneous real-time monitoring of both signals. Our methodology enables continuous monitoring of both $\text{O}_2^{\bullet-}$ and H_2O_2 produced by human neutrophil granulocytes. By suitable modifications the system can be even more versatile, *i.e.* by employing available biosensors for glutamate and nitric oxide detection (Chang et al., 2005a; O'Neill et al., 2004) and by using more specific and/or sensitive CL compounds, like isoluminol which is highly selective with respect to extracellular ROS only (Dahlgren and Karlsson, 1999; Lundqvist and Dahlgren, 1996). Hence, it would be possible to extend our approach for simultaneous, direct, quantitative real-time measurements of ROS (*e.g.*, $\text{O}_2^{\bullet-}$ and H_2O_2), nitrogenous species (*e.g.*, nitric oxide), and other messengers (*e.g.*, glutamate) produced by different cells types or even more complex biological systems. The experimental

set-up of the present approach (Fig. 1) could easily be expanded with additional biosensors or other CL compounds, provided the six-channel Biolumat is combined with a sensitive multi-channel potentiostat together with suitable software for multi-parameter data analysis.

In summary, the present work demonstrates a reasonably simple, moderately expensive, and extraordinarily powerful methodology to study the production of intra- and extracellular ROS from human neutrophils. It is also potentially useful in the study of other metabolites from different living cells or even tissues. The methodology might be a very useful tool in the research on the mechanisms of drug action and drug discovery processes, and would certainly contribute to the research of oxidative stress therapy.

Acknowledgements

The authors thank the European Community (contract QLK3-CT-2001-00244), the Swedish Research Council, the Swedish Society for Medicine, and the Goljes Minne, Magn Bergvall, Lars Hiertas Minne, and Nanna Svartz research foundations for their financial support. We also thank Dr. Zoltan Blum (Biomedical Lab Science, Health and Society, Malmö University) for critical reading and helpful suggestions.

References

- Allred, CD, Margetts, J, Hill, HR. Luminol-induced neutrophil chemiluminescence. *Biochim Biophys Acta* 1980; 631:380-85
- Alpeeva, IS, Niculescu-Nistor, M, Leon, JC, Csoeregi, E, Sakharov, IY. Palm tree peroxidase-based biosensor with unique characteristics for hydrogen peroxide monitoring. *Biosens Bioelectron* 2005; 21:742-48
- Amatore, C, Arbault, S, Bruce, D, de Oliveira, P, Erard, M, Vuillaume, M. Analysis of individual biochemical events based on artificial synapses using ultramicroelectrodes: cellular oxidative burst. *Faraday Discuss* 2000; 116:356
- Bainton, DF. Differentiation of human neutrophilic granulocytes: normal and abnormal. *Prog Clin Biol Res* 1977, 13:1-27
- Bamberg, R, Johnson, J. Segmented neutrophil size and platelet morphology in HIV/AIDS patients. *Clin Lab Sci* 2002;15:18-22
- Bard, AJ, Faulkner, LR. *Electrochemical Methods: Fundamentals and Applications*. Wiley, New York. 1980. 718 pp.
- Babior, BM. The participation of the hemes of flavocytochrome b245 in the electron transfer process in NADPH oxidase. *Blood* 1999; 93:4449
- Boyum, A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl* 1968; 97:77-89
- Campanella, L, Favero, G, Persi, L, Tomassetti, M. New biosensor for superoxide radical used to evidence molecules of biomedical and pharmaceutical interest having radical scavenging properties. *J Pharm Biomed Anal* 2000; 23:69-76
- Campanella, L, Favero, G, Tomassetti, M. A modified amperometric electrode for the determination of free radicals. *Sens Actuat* 1997; B44:559-65
- Chang, S-C, Pereira-Rodrigues, N, Henderson, JR, Cole, A, Bedioui, F, McNeil, CJ. An electrochemical sensor array system for the direct, simultaneous *in vitro* monitoring of nitric oxide and superoxide production by cultured cells. *Biosens Bioelectron* 2005a; 21:917-22
- Chang, S-C, Rodrigues, NP, Zurgil, N, Henderson, JR, Bedioui, F, McNeil, CJ, Deutsch, M. Simultaneous intra- and extracellular superoxide monitoring using an integrated optical and electrochemical sensor system. *Biochem Biophys Res Commun* 2005b; 327:979-84

- Cormier, MJ, Prichard, PM. An investigation of the mechanism of the luminescent peroxidation of luminol by stopped flow techniques. *J Biol Chem* 1968; 243:4706-14
- Dahlgren, C, Aniansson, H, Magnusson, KE. Pattern of formylmethionyl-leucyl-phenylalanine-induced luminol- and lucigenin-dependent chemiluminescence in human neutrophils. *Infect Immun* 1985; 47:326-28
- Dahlgren, C, Karlsson, A. Respiratory burst in human neutrophils. *J Immunol Methods* 1999; 232:3-14
- Emreguel, E. Development of a new biosensor for superoxide radicals. *Anal Bioanal Chem* 2005; 383:947-54
- Epstein, B, Kuwana, T. Electro-oxidation of phthalhydrazides. *J Electroanal Chem* 1967; 15:389-97
- Faldt, J, Ridell, M, Karlsson, A, Dahlgren, C. The phagocyte chemiluminescence paradox: luminol can act as an inhibitor of neutrophil NADPH-oxidase activity. *Luminescence* 1999; 14:153-60
- Faulkner, K, Fridovich, I. Luminol and lucigenin as detectors for superoxide radical. *Free Rad Biol Med* 1993; 15:447-51
- Ferapontova, EE, Grigorenko, VG, Egorov, AM, Borchers, T, Ruzgas, T, Gorton, L. Mediatorless biosensor for H₂O₂ based on recombinant forms of horseradish peroxidase directly adsorbed on polycrystalline gold. *Biosens Bioelectron* 2001; 16:147-57
- Ferrante, A, Thong, YH. Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leucocytes from human blood by the Hypaque-Ficoll method. *J Immunol Methods* 1980; 36:109-17
- Finkel, T. Signal transduction by reactive oxygen species in non-phagocytic cells. *J. Leukocyte Biol* 1999; 65:337-40
- Finkel, T, Holbrook, NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2000; 408:239-47
- Ge, B, Lisdat, F. Superoxide sensor based on cytochrome *c* immobilized on mixed-thiol SAM with a new calibration method. *Anal Chim Acta* 2002; 454:53-64
- Glass, GA, DeLisle, DM, DeTogni, P, Gabig, TG, Magee, BH, Markert, M, Babior, BM. The respiratory burst oxidase of human neutrophils. Further studies of the purified enzyme. *J Biol Chem* 1986; 261:13247-51
- Halliwell, B, Gutteridge, JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990; 186:1-85
- Krylov, AV, Adamzig, H, Walter, AD, Loechel, B, Kurth, E, Pulz, O, Szeponik, J, Wegerich, F, Lisdat, F. Parallel generation and detection of superoxide and hydrogen peroxide in a fluidic chip. *Sens Actuat* 2006; B119:118-26
- Kuwana, T. Electrooxidation followed by light emission. *J. Electroanal. Chem.* 1963; 6:164-67
- Lander, HM. An essential role for free radicals and derived species in signal transduction. *FASEB J* 1997; 11:118-

- Lindgren, A, Ruzgas, T, Gorton, L, Csoregi, E, Bautista Ardila, G, Sakharov, IY, Gazaryan, IG. Biosensors based on novel peroxidases with improved properties in direct and mediated electron transfer. *Biosens Bioelectron* 2000; 15:491-97
- Lisdat, F, Ge, B, Ehrentreich-Foerster, E, Reszka, R, Scheller, FW. Superoxide dismutase activity measurement using cytochrome c-modified electrode. *Anal Chem* 1999; 71:1359-65
- Lock, R, Johansson, A, Orselius, K, Dahlgren, C. Analysis of horseradish peroxidase-amplified chemiluminescence produced by human neutrophils reveals a role for the superoxide anion in the light emitting reaction. *Anal Biochem* 1988; 173:450-55
- Lucas, M, Solano, F. Coelenterazine is a superoxide anion-sensitive chemiluminescent probe: its usefulness in the assay of respiratory burst in neutrophils. *Anal Biochem* 1992; 206:273-77
- Lundqvist, H, Dahlgren, C. Isoluminol-enhanced chemiluminescence: a sensitive method to study the release of superoxide anion from human neutrophils. *Free Rad Biol Med* 1996; 20:785-92
- Lvovich, V, Scheeline, A. Amperometric sensors for simultaneous superoxide and hydrogen peroxide detection. *Anal Chem* 1997; 69:454-62
- Manning, P, McNeil, CJ, Cooper, JM, Hillhouse, EW. Direct, real-time sensing of free radical production by activated human glioblastoma cells. *Free Rad Biol Med* 1998; 24:1304-09
- McNeil, CJ, Athey, D, Ho, WO. Direct electron transfer bioelectronic interfaces: application to clinical analysis. *Biosens Bioelectron* 1995; 10:75-83
- McNeil, CJ, Greenough, KR, Weeks, PA, Self, CH, Cooper, JM. Electrochemical sensors for direct reagentless measurement of superoxide production by human neutrophils. *Free Rad Res Commun* 1992; 17:399-406
- McNeil, CJ, Manning, P. Sensor-based measurements of the role and interactions of free radicals in cellular systems. *Rev Mol Biotechnol* 2002; 82:443-55
- McNeil, CJ, Smith, KA, Bellavite, P, Bannister, JV. Application of the electrochemistry of cytochrome *c* to the measurement of superoxide radical production. *Free Rad Res Commun* 1989; 7:89-96
- O'Neill, RD, Chang, S-C, Lowry, JP, McNeil, CJ. Comparisons of platinum, gold, palladium and glassy carbon as electrode materials in the design of biosensors for glutamate. *Biosens Bioelectron* 2004; 19:1521-28
- Radi, R, Cosgrove, TP, Beckman, JS, Freeman, BA. Peroxynitrite-induced luminol chemiluminescence. *Biochem J* 1993; 290:51-57
- Roswell, DF, White, EH. The chemiluminescence of luminol and related hydrazides. *Methods Enzymol* 1978; 57:409-23
- Ruzgas, T, Csoregi, E, Emmneus, J, Gorton, L, Marko-Varga, G. Peroxidase-modified electrodes: fundamentals and application. *Anal Chim Acta* 1996; 330:123-38

- Scheller, W, Jin, W, Ehrentreich-Forster, E, Ge, B, Lisdat, F, Buttemeier, R, Wollenberger, U, Scheller, FW. Cytochrome c-based superoxide sensor for *in vivo* application. *Electroanalysis* 1999; 11:703-06
- Shipovskov, S, Ferapontova, EE, Gazaryan, I, Ruzgas, T. Recombinant horseradish peroxidase- and cytochrome c-based two-electrode system for detection of superoxide radicals. *Bioelectrochemistry* 2004; 63:277-80
- Shleev, S, Wetterö, J, Magnusson, K-E, Ruzgas, T. Electrochemical characterization and application of azurin-modified gold electrodes for detection of superoxide. *Biosens Bioelectron* 2006; 22:213-19
- Simchowitz, L, Textor, JA, Cragoe, EJ, Cell volume regulation in human neutrophils: 2-(Aminomethyl)phenols as chloride channel inhibitors. *Am J Physiol* 1993; 265:C143-C155
- Stief, TW. The blood fibrinolysis/deep-sea analogy. A hypothesis on the cell signals singlet oxygen/photons as natural antithrombotics. *Thrombosis Res* 2000; 99:1-20
- Tammeveski, K, Tenno, TT, Mashirin, AA, Hillhouse, EW, Manning, P, McNeil, CJ. Superoxide electrode based on covalently immobilized cytochrome c: modeling studies. *Free Rad Biol Med* 1998; 25:973-78
- Tarpey, MM, Fridovich, I. Methods of detection of vascular reactive species. Nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circulation Res* 2001; 89:224-36
- White, CR, Brock, TA, Chang, LY, Crapo, J, Briscoe, P, Ku, D, Bradley, WA, Gianturco, SH, Gore, J. Superoxide and peroxynitrite in atherosclerosis. *Proceed Nat Acad Sci USA* 1994; 91:1044-48
- Yamashita, K, Arai, T, Fukuda, K, Mori, H, Ishii, H, Nishioka, M, Tajima, K, Makino, K, Sasada, M. 6-formylpterin intracellularly generates hydrogen peroxide and restores the impaired bactericidal activity of human neutrophils. *Biochem Biophys Res Commun* 2001; 289:85-90
- Yao, D, Vlessidis, AG, Evmiridis, NP, Zhou, Y, Xu, S, Zhou, H. Novel chemiluminescence method for detection of superoxide anions and its application to dry-cured meat. *Anal Chim Acta* 2002; 467:145-53