Analytical Methods

DOI: 10.1002/anie.200702690

Indirect Electrochemical Sensing of Radicals and Radical Scavengers in Biological Matrices

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Free radicals, such as the hydroxyl radical $OH^{\bullet}$ and the superoxide radical $O_2^{\bullet-}$, belong to the most reactive chemical species known. In organic tissues, a number of enzymes, for example the superoxide dismutases, catalytically destroy these damaging species which are byproducts of the cell metabolism, and known for their potential carcinogenetic action.[1,2] Radicals also play an important role in the organism's innate immunity. Free oxygen radicals are produced by circulating monocytes and neutrophiles, in response to the lipopolysaccharide (LPS) of the Gram-negative bacterial outer membrane. This radical formation is important in the destruction of the bacteria after phagocytosis. The activation of these cells must be carefully regulated since excessive radical production may destroy the host's own tissue and contribute to septic shock.

So called antioxidants are compounds known to react with free radicals, inactivating them and thus preventing their cell-damaging action.[3,4] Because of the great importance of free radicals and antioxidants, there is considerable demand for techniques to detect and quantify these two groups of compounds. Electron spin resonance ranks first for radical detection because it is highly selective for the detection of paramagnetic species.[5] In some cases UV/Vis spectroscopy can also be applied to detect certain free radicals.[6] Antioxidants on the other hand are usually quantified by their destructive action towards free radicals. Whereas spectroscopic techniques can be highly selective and sensitive for certain radicals, this is not true in all cases and it is frequently difficult to apply them in situ in chemical or biological systems. A number of highly sensitive electrochemical biosensors for detection of free oxygen radicals and antioxidants have been reported.[7–11] They are based on the use of immobilized redox proteins, especially cytochrome C, which is easily reduced by the oxygen radicals, and typical catalytic currents can be measured. All these protein-based biosensors suffer from a limited stability, and their preparation is rather time consuming.

Herein, we report a completely new approach to detect free radicals using an electrochemical procedure, in which the radicals destroy a well defined molecular layer on an electrode. Self-assembled monolayers (SAMs) of alkylthiols can be easily prepared on the surface of mercury and gold electrodes, and, if suitable compounds are adsorbed, the electrochemical signal of a dissolved redox probe, for example, the hexammine ruthenium(III) complex, can be completely blocked.[12,13] Although these SAMs are known to be stable, we have discovered that they can be rather easily attacked by free radicals. When such an electrode with a SAM is exposed to a solution in which free radicals are generated, for example, by the Fenton reaction [Eq. (1), see for example ref. [14–16]], the free radicals destroy the SAM, and the electrochemical signal of the redox probe recovers to a degree proportional to the extent of dissolution of the SAM.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (1)$$

Figure 1 depicts voltammograms recorded at a mercury electrode and at a gold electrode in a solution of $[Ru(NH_3)_6]^{3+}$ before modification of the electrode surface with a SAM of hexanethiol, after SAM formation, and after attacking the SAM with $OH^{\bullet}$ radicals produced in a Fenton solution for 1 minute or for 5 minutes. Figure 2 shows a plot of peak currents versus time of reaction of the SAM with the $OH^{\bullet}$ radicals of the Fenton solution. Control experiments have shown that the SAM is neither attacked by the hydrogen peroxide, nor by iron(II) or iron(III) ions. Experiments with hexacyanoferrate(II) as redox probe and hexanethiol SAM on gold and hexadecanethiol SAMs on gold electrodes revealed that the hexadecanethiol is also attacked by the $OH^{\bullet}$ radicals, although complete removal of the SAM could not be achieved. The removal of SAMs from the electrode can be also achieved by very strong oxidants, such as permanganate in acidic solution; however, such reactions cannot

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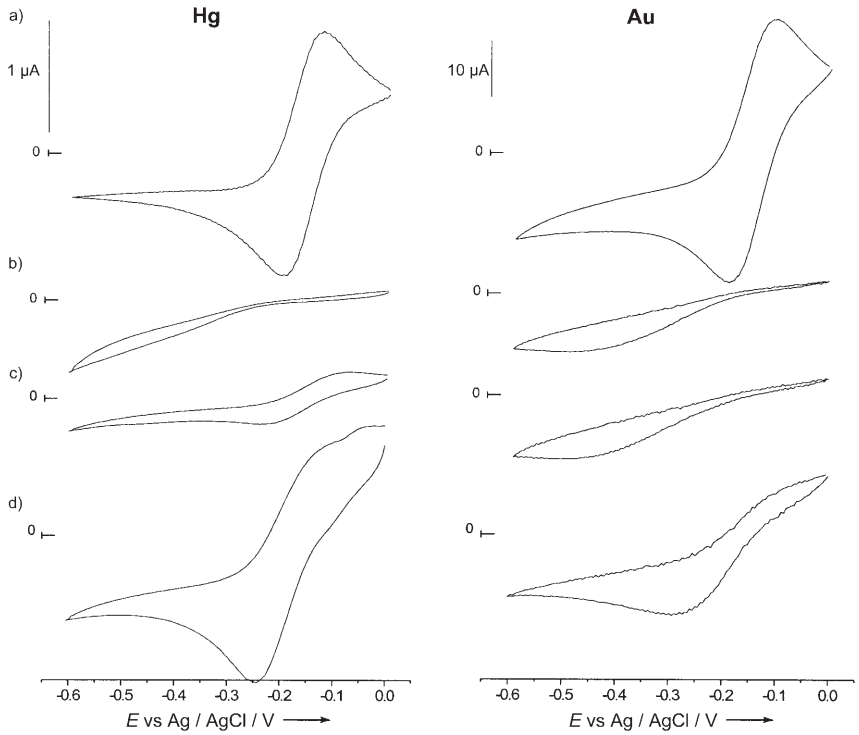

Figure 1. Cyclic voltammograms (CVs) of a $[Ru(NH_3)_6]^{3+}$ (1×10^{-3} mol L^{-1}) solution recorded with unmodified electrodes (a), SAM (hexanethiol) modified electrodes (b), and SAM-modified electrodes after attacking the SAM with a Fenton solution for 1 min (c), and for 5 min (d). CVs recorded in 1×10^{-3} M $[Ru(NH_3)_6]Cl_3$ in 0.01 M acetate buffer. Scan rate 500 mV s^{-1}.

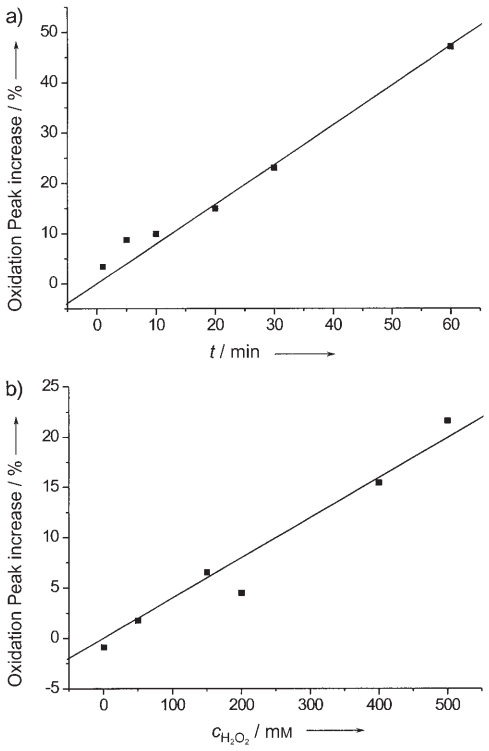

Figure 2. Dependence of the increase of oxidation peak a) on time of exposure of the SAM (hexanethiol) modified electrode to Fenton solution, and b) on the of concentration hydrogen peroxide, keeping exposure time constant (5 min). Composition of Fenton solution for (a): 0.1 mol L^{-1} Fe^{2+} and 0.1 mol L^{-1} H_2O_2, pH 4.7; for (b): the molar ratio of Fe^{2+} to H_2O_2 was kept 1:1. CVs were recorded as for Figure 1.

proceed under physiological conditions, so that their competition need not to be taken into account in a biological setting. It is of note that in the experiments with the above mentioned biosensors, the self-assembled monolayers used to immobilize the redox proteins are not deteriorated by oxygen radicals. This situation might be due to the highly stable SAM compounds used, for example, 11-mercaptoundecanoic acid, and certainly also due to very low radical concentrations. Doubtless, the right choice of SAMs will decide on the extent of deterioration of the SAM by oxygen radicals.

This new approach to quantify the action of free radicals by their destruction of a SAM on an electrode surface can be also applied for quantification of antioxidants, that is, scavengers of free radicals. This processes is shown by the addition of ascorbic acid to the Fenton solution, which leads to an easily detectable decrease of the destructive power of the Fenton solution (Figure 3).

A first attempt was also made to detect the free radicals formed when human peripheral blood mononuclear cells (PBMC) were treated with the lipopolysaccharides (LPS) from Gram-negative bacteria, a treatment performed to provoke the production of reactive oxygen species (oxygen burst). The PBMC were suspended at a cell density of 3×10^6 mL^{-1} in RPMI 1640 culture medium supplemented with penicillin and streptomycin and with 10% foetal calf serum. LPS isolated from *Salmonella typhimurium* was added to final concentrations of 20, 200, or 1000 ng mL^{-1}. The time of exposure of the electrode to the activated cells was varied from 5 to 30 minutes. The recovery of the ruthenium signal ranged between 0.4% (for a 5 min exposure to 200 ng mL^{-1} LPS) and 9.6% (for a 20 min exposure to 200 ng mL^{-1} LPS). For a

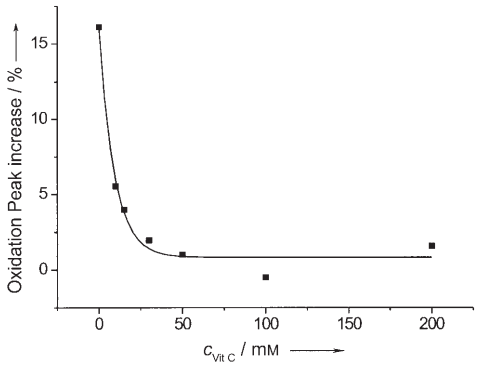

Figure 3. Dependence of the increase of the oxidation peak of $[Ru(NH_3)_6]^{2+}$ after 5 min of exposure to the Fenton solution ($c_{Fe^{2+}}$ and $c_{H_2O_2}=0.2$ mol L^{-1}, pH 4.7) on the concentration of ascorbic acid $c_{Vit\,C}$ in the Fenton solution. CVs recorded as for Figure 1.

sample of unfractionated whole blood and 200 ng mL^{-1} LPS the recovery increased from 0.2% at 5 minutes exposure time, to 0.7% at 10 minutes, and finally to 0.9% at 20 minutes. With a constant exposure time of 5 minutes the recovery increased from 0.2% for 200 ng mL^{-1} LPS to 1.4% for 1000 ng mL^{-1} LPS. The recovery of the ruthenium signal achieved was equivalent to that seen following the action of Fenton solutions with concentrations of hydrogen peroxide in the millimolar range. Thus, the effect of 20 ng mL^{-1} LPS, on PBMC and a 5 minutes exposure time was as strong as the action of a Fenton solution with 4 mmol L^{-1} H_2O_2. Control experiments showed that the signal recovered was entirely confined to experiments with living blood cells.

The experiments reported herein demonstrate that irreversibly adsorbed layers on electrode surfaces can be used in studies of free radicals, and also for quantifying the radical scavenging action of antioxidants. With our system, the 3σ-detection limit for detection of H_2O_2 is 10^{-5} mol L^{-1} for 5 minutes exposure time. Given the very short life time of the OH radicals, their concentration will be considerable smaller.

It is to be expected that various other layers, such as dyes, drugs, and polymers, will be useful in such studies. Preliminary experiments with adsorbed riboflavine are very promising, especially also with respect to much lower detection limits. Adsorbed riboflavine has the advantage of being electroactive, so that no other redox probe is needed. The use of modified gold nanoelectrodes may even allow in situ measurements of radical activities in living tissues.

Experimental Section

A gold disc electrode (3 mm diameter) and a mercury multimode electrode (both from Metrohm, Herisau) were used as working electrodes. The counter electrode was a thick platinum wire, the reference electrode was a Ag/AgCl in 3 M KCl. All measurements were performed using a μ-AUTOLAB III (Eco-Chemie, Utrecht). Hexanethiol and decahexanethiol were from Aldrich. The Fenton solution was always freshly prepared from ammoium iron(II) sulfate hexahydrate (Merck) or from iron(II) chloride tetrahydrate and iron(II) sulfate heptahydrate (Merck), and a 0.01 M acetate buffer (pH 4.7) and hydrogen peroxide solution. The Fenton solution was prepared using different concentrations of both Fe^{2+} and H_2O_2, ranging between 0 and 500 mmol L^{-1}. Unless otherwise indicated, the molar ratio of Fe^{2+}:H_2O_2 was 1:1. The gold electrode was polished prior to the experiments as described elsewhere.[17] The SAM monolayers on the electrodes were prepared from an ethanolic solution containing the alkanethiol in a ratio 20% (v/v) according to a method described elsewhere.[18,19] The so-called "soaking procedure" was employed. Briefly, the Hg drop was immersed in a 20% (v/v) solution of the alkanethiol in ethanol (EtOH) for a short time (15 s to 2 min in the case of mercury electrodes; 5 min for gold electrodes). The thiol-coated electrode was washed with pure ethanol and water, and finally introduced to the $[Ru(NH_3)_6]^{3+}$ chloride solution (1×10^{-3} mol L^{-1}, ACROS), a cyclic voltammogram was then recorded. The electrode was washed with water and introduced to a freshly prepared Fenton solution for a defined time interval (1 to 60 min). The reaction of the Fenton solution with the SAM-modified electrode was terminated by removing the electrode from the Fenton solution and by washing with water and pure ethanol. The result of the Fenton attack was finally probed by measuring the redox probe $[Ru(NH_3)_6]^{3+}$ or hexacyanoferrate(II) with the same electrode. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll (1.077 g mL^{-1}) as previously described.[20] RPMI 1640 culture medium, penicillin/streptomycin solution and foetal calf serum were all purchased from Gibco-Invitrogen. Lipopolysaccharide purified from *Salmonella typhimurium* was from Sigma Aldrich.

Received: June 19, 2007
Revised: July 10, 2007
Published online: September 17, 2007

Keywords: analytical methods · antioxidants · electrochemistry · radicals · ruthenium

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