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Electrochemical microsensors for cutaneous surface analysis: Application to the determination of pH and the antioxidant properties of *stratum corneum*

Microcapteurs électrochimiques pour l'analyse de la surface cutanée : applications à la détermination du pH et des propriétés antioxydantes du *stratum corneum*

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Abstract

Potentiometry and cyclic voltammetry were proposed as simple, reliable and non invasive methods for the simultaneous determination of pH and antioxidant properties of skin. Experiments were performed with microelectrodes just deposited on skin surface without any gel or water added. pH was measured by means of the zero current potential of a tungsten W/WO₃ sensor. A Nernstian response was recorded in pH range 4 to 6 corresponding to the normal skin pH values. The global antioxidant capacity was deduced from the anodic charge passed during the plotting of cyclic voltammograms on platinum or gold microelectrodes. Comparing the half wave or peak potentials of these curves with those recorded for experiments performed in aqueous solution, the main hydrophilic antioxidants species were detected, i.e. ascorbic acid, uric acid and glutathione. This relatively easy-to-use analytical method made it possible to follow in real time the efficiency of topical treatment as well as to study the influence of oxidative stress.

Résumé

La potentiométrie et la voltammétrie cyclique sont proposées comme des méthodes simples, précises et non invasives pour la détermination simultanée du pH et des propriétés antioxydantes de la peau. Les expériences sont réalisées avec des microélectrodes posées à la surface de la peau, sans addition de gel ou d'eau. Le pH est obtenu par la mesure du potentiel à courant nul d'un capteur à tungstène W/WO₃. Une réponse Nernstienne est obtenue dans une gamme de pH de 4 à 6 correspondant au pH de la peau saine. La capacité antioxydante globale est déduite de la quantité de charge anodique au cours de tracé de voltammogrammes sur microélectrodes de platine ou d'or. En comparant les potentiels de demi-vague ou les potentiels de pic de ces courbes avec ceux issus d'expériences réalisées en solution aqueuse, les principales espèces antioxydantes hydrophiles sont détectées (acide ascorbique, acide urique, glutathion). Cette méthode d'analyse, relativement simple d'utilisation, permet de réaliser en temps réel le suivi d'un traitement topique mais aussi d'étudier l'influence du stress oxydant.

Keywords: Antioxidants; Cyclic voltammetry; Microelectrodes; pH; Skin

Mots clés : Antioxydants ; Microélectrodes ; Peau ; pH ; Voltammétrie cyclique

1. Introduction

Human skin is a complex organ that plays important protecting and regulating functions. It is composed of three morphologically distinct tissues: hypodermis, dermis and the

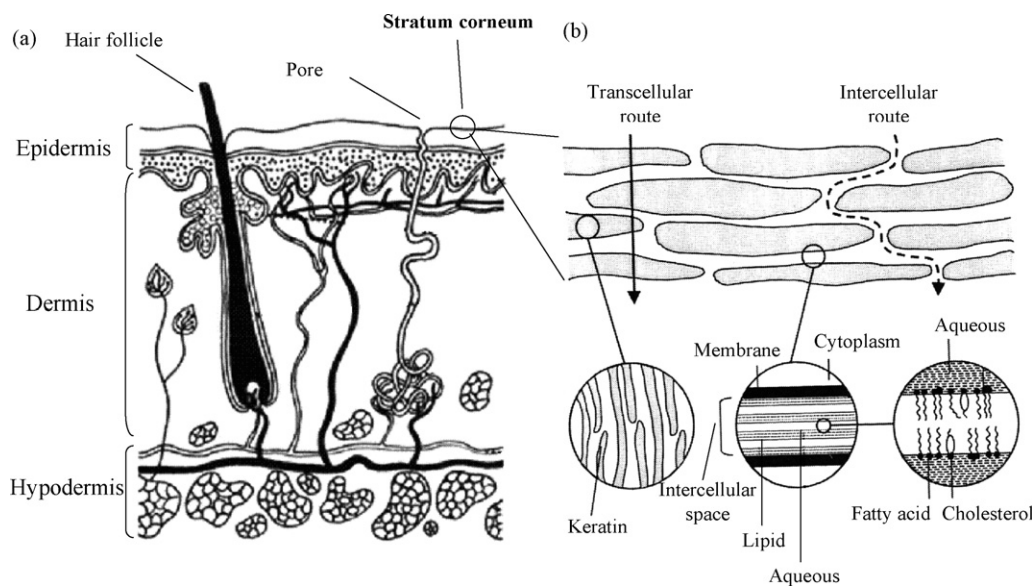


Fig. 1. (a) General structure of the skin. (b) Diffusion pathways through the *stratum corneum* (adapted from [2]).

outer epidermis (Fig. 1a). The epidermis has a stratified structure that corresponds to the different steps of cell differentiation. Moving towards outside from the proliferative basal layer, the cells change from metabolically active and dividing cells to functionally dead and keratinized cells called corneocytes. These cells constitute the outer layer of epidermis, the *stratum corneum*, whose thickness is between 10 and 50 μm [1]. *Stratum corneum* constitutes the primary skin diffusion controlling barrier and its properties are based on a specific composition. The corneocytes are strongly sealed in an extracellular matrix enriched by nonpolar lipids (ceramides 50%, free fatty acids 25%, free cholesterol 20%) showing an exceptional structural arrangement (Fig. 1b). Moreover, a fine hydro-lipidic emulsion (between 0.5 and 5 μm thick), called cutaneous surface film, recovers the *stratum corneum* and penetrates between the corneocytes. Thus, skin can be considered as a nanoporous barrier controlling the diffusion of molecules according to distinct pathways: through the appendages (i.e. hair follicles and sweat glands), through an intercellular route between the corneocytes and through a transcellular route crossing the corneocytes as illustrated Fig. 1b [2].

Since several years, the development of many cutaneous analysis techniques has made it possible to advance in the comprehension of the anatomical, physicochemical and functional characteristics of a normal skin. These methods have also brought qualitative and quantitative information on the physiological as well as pathological processes and have allowed to appreciate the effects of drugs and dermocosmetics products [3]. A particular effort has been done for skin ageing study. Skin represents a major target of oxidative stress since it is continuously exposed to external aggressions like ultraviolet radiation (UV), ozone or chemicals. Repeated exposures to such aggressions result in skin premature ageing and contribute to the development of cutaneous dermatoses and cancers [4]. A wide variety of analytical methods for evaluating oxidative stress have been proposed. They are able to determine the quantity of reactive oxygen

species, antioxidants or oxidation products. Measurements are usually performed by electron spin resonance, chemiluminescence, chromatography, spectroscopy and mass spectrometry [5]. Nevertheless, these techniques require expensive equipments, involve complex protocol, often need skin biopsy and provide only delayed results. Electrochemical methods were recently adapted and developed in order to define an indicator of antioxidant global properties of real samples like wine, biological fluids or tissues [6–8]. The most frequently used techniques concern potentiometric titration, cyclic voltammetry or electrochemical sensors. Kohen recently applied this technique successfully to skin analysis [9,10]; nevertheless the method used was either invasive because it involved skin homogenates or indirect as it was performed in an electrolytic solution in contact with the skin surface. Moreover, these measurements carried out with macroelectrodes (surface areas in the range of a square centimeter) presented low sensitivity and did not allow localized measurements.

In other respects, skin pH measurement is essential for biochemists and dermatologists since surface acidity is an important part of the cutaneous ecosystem and is involved as a defense system against microbiological or chemical aggressions. Modifications of pH can reflect or induce changes in the activity of several enzymes; consequently skin's acidity plays an important role in barrier homeostasis and in *stratum corneum* desquamation [3]. Moreover, pH measurements are essential to characterize diseases, like atopic dermatitis or xeroderma, or to evaluate treatment efficiency [11,12]. Usually quoted in the bibliography and commercially available in many models, glass membrane electrode is traditionally used for skin pH determination whose values are generally between 4 and 6. But this conventional glass electrode has also its obvious drawbacks, such as the temperature dependence, the fragility of the glass membrane and the limited potentialities of miniaturization. Moreover, these electrodes are not appropriate for in situ measurement in a complex media, particularly in non aque-

ous medium. Since several years, different pH sensors based on metal/metal oxide electrodes were developed because such electrodes are robust in structure, present a short response time and are less sensitive to cations interference. Furthermore, they offer excellent characteristics of easy miniaturization [13,14].

In this context, we have developed for a few years the application of some electrochemical methods for the analysis of skin antioxidant properties and pH. Preliminary works have shown the possibility to perform electrochemical measurements on the human epidermis surface by means of microelectrodes [15,16]. They allowed to realize non-invasive, direct (without adding water or gel), rapid and reliable measurements with high spatiotemporal resolution. Both pH and the overall redox status of the skin were determined by recording cyclic voltammograms with platinum microelectrode. In order to avoid possible interactions between both measurements, the determination of pH and the antioxidant properties of skin is here performed simultaneously by means of several distinct microelectrodes [17]. On one hand, skin pH was determined potentiometrically on tungsten. Tungsten was chosen because of its relatively low cost compared to noble metals. Furthermore, tungsten oxides are easily obtained by electrochemical oxidation of the metal at low potential and are strongly stable. On the other hand, cyclic voltammetry using platinum and gold microelectrodes was used for the analysis of the *stratum corneum* antioxidant capacities. Determination of the nature of main antioxidant species involved is also reported. The paper finally presents results of the first preclinical studies highlighting the evolution of the amperometric response with time or the influence of a topic treatment including ascorbic acid. Also, included results obtained in the case of a skin eczema or in the case of a continuous exposition to external aggressions.

2. Materials and methods

2.1. Reagents

All chemicals were reagent grade and used as received. Potassium ferricyanide $K_3[Fe(CN)_6]$ and potassium chloride KCl were purchased from Acros Organics (France). Potassium dihydrogen phosphate KH_2PO_4 and dipotassium hydrogen phosphate K_2HPO_4 were purchased from Sigma-Aldrich (France). The two phosphate solutions were prepared at a concentration of 0.1 mol L^{-1} and were mixed to obtain different buffer solutions with appropriate pH. The ferricyanide solution was prepared in a phosphate buffer KH_2PO_4/K_2HPO_4 0.1 mol L^{-1} , pH 7. For chromatographic experiments, ethylene diamine tetraacetic acid was purchased from Acros Organics whereas β -mercaptoethanol was purchased from Sigma-Aldrich. The dermocosmetic cream Active C was from La Roche-Posay (France).

2.2. Electrochemical instrumentation

Electrochemical experiments were carried out at room temperature with an Autolab Metrohm potentiostat controlled by the General Purpose Electrochemical System software (Metrohm, France). When several microelectrodes were used simultane-

ously, a VMP2/Z multipotentiostat was involved, controlled by the ECLab software (Ametek, France). The electrode system consisted of a saturated calomel electrode (SCE) as reference, a large-surface-area platinum electrode as counter and one or several hand-made disk microelectrodes as working electrodes. Microelectrodes were made with $50 \text{ }\mu\text{m}$ diameter platinum, gold and tungsten wires (Ref. PT025110, AU005125 and W005135, respectively, Goodfellow, France) inserted in a glass capillary (GC-120F-10, Clark Electromedical Instruments, Phymep, France). For some comparative data, a 1 mm diameter platinum wire (Ref. LS263293, Goodfellow, France) was also used as working electrode.

2.3. Fabrication and electrochemical characterization of microelectrodes

The $50 \text{ }\mu\text{m}$ diameter metallic wire was inserted in a glass capillary. It was pulled in two microelectrodes with a microelectrode puller (Model PC10, Narishige, U.K.). After strengthening the glass and metal by heating, the tip was polished on the diamond particle whetstone microgrinder (Model EG44, Narishige, U.K.) for several minutes. The resulting microelectrodes presented a disk geometry with a total radius of less than $120 \text{ }\mu\text{m}$ [15].

Electrochemical characterization of each microelectrode was performed by plotting a cyclic voltammogram in a 5 mmol L^{-1} deaerated ferricyanide solution between -0.5 V and 0.4 V/SCE for platinum and gold microelectrodes or between -0.8 V and -0.2 V/SCE for tungsten microelectrodes. A steady state curve was rapidly obtained and the limiting current recorded at around -0.4 V/SCE is directly proportional to the disk radius:

$$I_{\text{lim}} = 4nFrDC$$

where I_{lim} is the limiting current (A), n is the number of electrons exchanged, F is Faraday's constant (96500 C mol^{-1}), D is the diffusion coefficient ($\text{cm}^2 \text{ s}^{-1}$), C is the concentration of the electroactive specie (mol cm^{-3}) and r is the metallic wire radius (cm) [18]. More than 300 microelectrodes radius were calculated using this relation. The average value was $30 \pm 3 \text{ }\mu\text{m}$ (i.e. an accuracy of 10%). This value was coherent with the wire radius commercially indicated and showed the good repeatability of the microelectrode fabrication. In addition, more than 100 reproducible cyclic voltammograms were recorded successively in the ferricyanide solution. Consequently the adopted protocol is a simple technique and presents a good reproducibility for microelectrodes fabrication and their electrochemical responses.

2.4. Preparation of metal/metal oxide electrodes

Electrochemical oxidation of tungsten in aqueous electrolyte results in tungsten oxides, mainly WO_3 . This arises at potentials around $0.2\text{--}0.4 \text{ V/ESH}$ in pH range from 4 to 7. pH sensor based on W/WO_3 electrode was prepared by constant potential electrolysis of bare electrode at 1 V/SCE in an aerated phosphate buffer pH 7 during two minutes. This protocol induced further stable potentiometric measurements in short time, i.e. less than two minutes.

2.5. *In vivo* measurements

Otherwise indicated, electrochemical measurements were realized on the inside of the forearm of volunteers. Reference electrode, counter-electrode and one or several working microelectrodes were placed directly on the skin surface without adding water or gel. The saturated calomel reference electrode was connected to the skin by a felt-tip pen used as a Luggin capillary. This particular device also avoided the diffusion of the internal saturated chloride solution of the electrode through the skin.

Potentiometric measurements were achieved with W/WO₃ sensors by recording the potential at zero current. Reference measurements of skin pH were made by applying a glass membrane electrode on the inside of the forearm (skin-pH-meter pH 900 Courage and Khazaka, Monaderm, France). In order to compare all the results with the same experimental conditions, all electrochemical measurements were carried out without adding water.

Cyclic voltammograms were plotted at 50 mV s⁻¹ between -0.4 V and 1.2 V/SCE on platinum microelectrodes or between -0.3 V and 1.5 V/SCE on gold microelectrodes. In each experiment, the lower and upper potentials were chosen in order to avoid oxidation and reduction of water, which could modify the medium pH. In order to compare the results obtained on the skin with different microelectrodes, all the amperometric responses were referenced to the same surface area of the electrode.

2.6. HPLC analysis

Chromatographic determination of ascorbic acid on skin was as following: cutaneous removals were carried out by friction of a cotton stem soaked with a removal buffer (phosphate buffer 50 mmol L⁻¹ pH 3.0 + EDTA 1 mmol L⁻¹) on a skin area of 10 cm². The stem tip was cut and placed in an eppendorf tube with an extraction buffer (phosphate buffer 50 mmol L⁻¹ pH 3.0 + EDTA 1 mmol L⁻¹ + β-mercaptoethanol 10 mmol L⁻¹). After vortex (30 s), a 150 μL aliquot was analyzed by high performance liquid chromatography (HPLC) involving an Agilent HPLC system and an ODS Hypersyl C18 column (4.6 × 200 mm, 5 μm). Elution was realized with the extraction buffer (isocratic mode) with a flow rate of 0.8 mL min⁻¹ from 0 to 11 min, 1.6 mL min⁻¹ from 12 to 29 min and 0.8 mL min⁻¹ from 30 to 35 min. Ascorbic acid was detected by constant potential electrolysis on a vitreous carbon electrode at 0.6 V/Ag/AgCl. In order to counterbalance the different strengths applied during the frictions and so to compare the different samples, ortho-phthalaldehyde (OPA) fluorescent protein assays were performed simultaneously.

3. Results and discussion

3.1. Potentiometric measurements of skin pH

3.1.1. With a glass membrane electrode

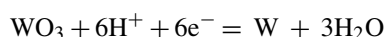
Glass membrane electrode is traditionally used for skin pH determination because of its high selectivity, stability, and wide

pH range. This potentiometric method is really simple; subjects are relaxed in a controlled atmosphere room (temperature, humidity) and the electrode is only deposited on their epidermis surface. pH determination is currently obtained in 5 to 30 s with a precision of ±0.1 pH [19,20]. Nevertheless measurements are often carried out with distilled water film deposited on skin surface [3,21]; the addition of water to the interface electrode/skin can have a non negligible effect on the pH value.

This effect was highlighted during a preclinical study involving a group of 24 subjects. Two types of measurements were carried out: one with the glass electrode directly deposited on skin, the other with 200 μL distilled water previously put on skin. The average values and standard deviation obtained on each arm were determined. These results showed a decrease of 0.8 pH unit when experiment was performed with water. However, absence of water did not seem to disturb the measurement; the standard deviation was even smaller in this case. This evolution was the same whatever the arm involved. This experiment clearly shows that settle a drop of water on the skin before the electrochemical measurement involves a significant pH modification. This could result from the relatively high absorption rate of atmospheric carbon dioxide into the distilled water which decreased sensitively the resulting pH. Moreover, the hydrolipidic film present on the skin surface is thus modified: adding water liberates protons from free fatty-acids and induces diffusion of the elements of Natural Moisturizing Factor (NMF) as lactic and pyruvic acids, resulting in a more acidic pH [3]. Glass membrane electrode was later used as reference. In order to compare all the results with the same experimental conditions, all electrochemical measurements were further carried out without adding water.

3.1.2. With a W/WO₃ electrode

Considering WO₃ as the main oxide resulting from the electrochemical oxidation of tungsten, the oxidoreduction reaction occurring at the interface in equilibrium state is:



Assuming all activities equal to 1, the Nernst's equation is:

$$\begin{aligned} E_{(t=0)} &= E_{\text{O}}(\text{WO}_3/\text{W}) + \frac{RT}{F} \ln[\text{H}^+] \\ &= E_{\text{O}}(\text{WO}_3/\text{W}) - 0.059 \text{ pH at } 298 \text{ K} \end{aligned}$$

At a given temperature, the equilibrium potential of a metal/metal oxide electrode depends finally only on pH. Fig. 2 shows the correlation between the zero current potential measured with the W/WO₃ electrode and the cutaneous surface pH measured with a glass membrane electrode in the same experimental conditions, particularly the same room temperature. On this figure is also reported the calibration curve obtained in phosphate buffer solutions with different pH values. The first correlation showed a good linear relationship between the electrode potential and the epidermis pH; the slope was 64 mV/pH unit, close to the theoretical Nernstian response. Moreover, the calibration curve was very similar to that obtained in buffer solution. This last result proves the validity of the analytical process tested in a relatively complex medium.

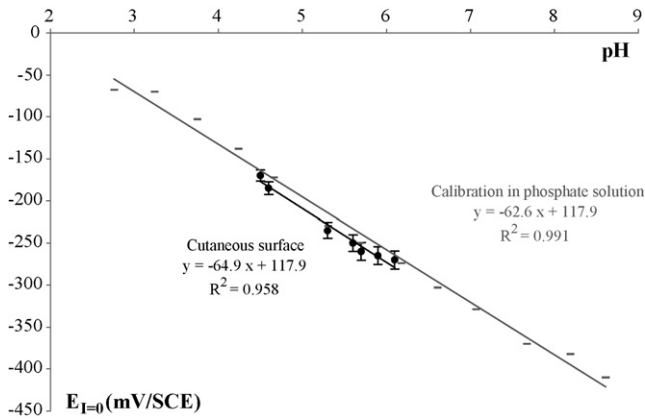


Fig. 2. Correlation between the zero current potential measured with a W/WO_3 sensor and the pH measured with a glass membrane electrode, in phosphate solutions (-) and on cutaneous surfaces (●) (10 volunteers). Each potential value corresponds to the average of 10 measurements realized with different microelectrodes in a 1 cm^2 skin area.

3.2. Amperometric measurement

3.2.1. Cyclic voltammetry on skin

Direct electrochemical measurements were performed on the skin surface with a 1 mm diameter-disk platinum electrode and with a $50 \mu\text{m}$ diameter-disk platinum microelectrode. Using a macroelectrode, the corresponding cyclic voltammogram displayed a current with high resistive and capacitive components (Fig. 3a), leading to unusable data. In comparison, a well defined sigmoid steady-state voltammogram was obtained with the microelectrode showing different anodic and cathodic current waves. (Fig. 3b). The miniaturized size of the working microelectrode enhanced mass transport conditions in the vicinity of the electrode surface, thus improving the sensitivity of the response. Moreover, the current generally recorded being in the range of a few nano-amperes, resistive and capacitive currents are minimized compared to the faradic component [22]. It was verified that the current-potential curves were identical whether the experiments were performed with or without water. It was thus been considered that applying the microelectrode directly on the surface would give more reliable results than adding water able to modify the medium.

The guidelines drawing (Fig. 3) allows to estimate the resistance R between reference and working electrodes according to the Ohm's law. It is correlated to resistivity ρ : $R = \rho(l/S)$, where l is the distance between the two electrodes (cm). This value was overestimated to 1 cm, the electrodes being located at 0.5–1 cm of each other. Table 1 summarized results calcu-

Table 1
Electrical resistivity of *stratum corneum* determined with macroelectrode and microelectrode

	Macroelectrode	Microelectrode
Surface (cm^2)	3.14×10^{-2}	1.96×10^{-5}
Resistance		
$R = \Delta E / \Delta I$ (Ω)	5.2×10^6	3.2×10^9
Resistivity ($10^3 \Omega \text{ cm}$)	163	63

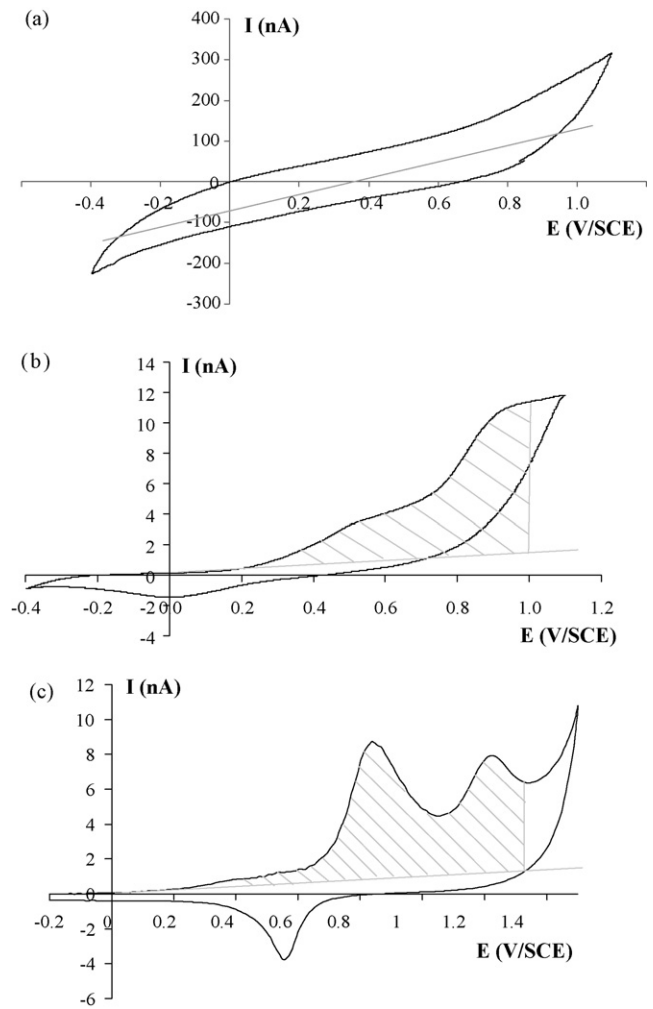


Fig. 3. Cyclic voltammograms obtained directly on the skin surface (a) with a 1 mm diameter platinum electrode. (b) with a $50 \mu\text{m}$ diameter platinum electrode. (c) with a $50 \mu\text{m}$ diameter gold electrode. Potential scan rate: 50 mV s^{-1} .

lated for the two sizes of electrode. Even though the medium was the same, the resistivity for a microelectrode was more than two times lower. It should be noted that this equation considers an electrolytic solution column with a section S and a length l . This simplified model was not totally adapted to the case of macroelectrodes. Using the result obtained with the microelectrode, it was possible to estimate the specific conductivity $\sigma = 1/\rho$ of the skin around $1.6 \times 10^{-5} \Omega^{-1} \text{ cm}^{-1}$. This value is close to that previously estimated by conductance or impedance measurements, around $1 \times 10^{-5} \Omega^{-1} \text{ cm}^{-1}$ [3]. This value can also be compared to those of classic media, like distilled water ($4 \times 10^{-8} \Omega^{-1} \text{ cm}^{-1}$) or $\text{KCl } 0.1 \text{ mol L}^{-1}$ often used as electrolyte ($1.3 \times 10^{-2} \Omega^{-1} \text{ cm}^{-1}$) indicating that the cutaneous surface is a relatively good conducting medium owing to the presence of a lot of mineral salts in the aqueous phase of the cutaneous surface film.

3.2.2. Metrological specifications

When successive cyclic voltammograms was performed with the same microelectrode, the anodic currents recorded decreased as scan number increased, whether the microelectrode was

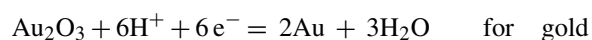
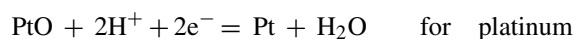
maintained on the same surface area or not. These variations certainly resulted from a modification of the electrode surface state induced by the electrochemical oxidation of the antioxidant species [16]. Consequently, the evaluation of repeatability, by means of the standard deviation of the signal obtained with the same electrode placed on the same skin surface, was therefore impossible and our microelectrodes were single use.

In order to validate the methodology, a multipotentiostat was used to determine the accuracy of the measurement. The electrochemical cell included a saturated calomel reference electrode, a platinum counter-electrode and three working microelectrodes for simultaneously recording three cyclic voltammograms. For these measurements, 20 volunteer subjects were involved and a set of three new microelectrodes was used for each subject. The mean standard deviations was estimated to 12% for the current recorded at 0.9 V/SCE at platinum microelectrodes and to 18% and 15% for the currents recorded at gold microelectrodes at 0.8 and 1.2 V/SCE, respectively (see below). These results attest the reliability of the process.

3.3. Antioxidant properties evaluation

3.3.1. Global antioxidant capacity

Fig. 3b and c show cyclic voltammograms obtained on the skin surface with a 50 μm diameter platinum microelectrode and with 50 μm diameter gold microelectrode, respectively. These two voltammograms presented a cathodic current peak (at around 0 V and 0.6 V/SCE, respectively) corresponding to the metal oxide reduction:



The anodic parts of these voltammograms showed two waves with limiting currents recorded at 0.6 V and 0.9 V/SCE for platinum microelectrodes on one hand and a first wave from 0.2 V/SCE followed by two peaks at 0.8 and 1.2 V/SCE for gold microelectrodes on the other hand. These anodic currents are related to electrochemical oxidation reactions which are due to the presence of reduced species at the electrode/skin interface. After correction of the residual current estimated by the extrapolation of the tangent at 0 V/SCE as shown in Fig. 3, the *stratum corneum* global antioxidant capacity was estimated through the maximum anodic current or the total anodic charge: $Q = \int_0^t I \cdot dt$ (hatched area).

Complementary experiments were performed by immersing platinum and gold microelectrodes in 0.1 mol L⁻¹ phosphate buffer or 20 mmol L⁻¹ potassium chloride solutions (close to sweat chloride concentration). The anodic charge corresponding to the metal oxidation and to the oxidation of chloride ions were recorded (data not shown). It was verified that these amounts of charge was less than 30% of the anodic charge recorded when the experiment was performed directly on the skin surface. Consequently, the anodic responses recorded on the cutaneous surface were principally correlated to reduced species acting as antioxidants.

3.3.2. Electrochemical detection of major hydrophilic antioxidant species

Several experiments were performed in order to determine the main hydrophilic antioxidant species detectable with such a simple non invasive electrochemical method. Firstly, cyclic voltammograms were recorded with a platinum microelectrode in ascorbic acid or uric acid 1 mmol L⁻¹ pH 5.0 solutions. The half wave potentials $E_{1/2}$ (i.e. the potential of the curve where the current is half the diffusion limited current) were 0.4 V and 0.8 V/SCE, respectively. A similar experiment was realized with a gold microelectrode in glutathione 1 mmol L⁻¹ pH 5.0 solution. It is more relevant to use gold in this later case because of the well-known spontaneous self-assembled thiol monolayers taking place on such material. In these conditions $E_{1/2}$ was 1.1 V/SCE. Secondly, cyclic voltammograms were recorded on an untreated skin area and on a treated area as following: 50 μL of an aqueous solution pH 5.0 containing ascorbic acid, uric acid or glutathione 0.1 mol L⁻¹ was simply deposited on the skin surface during five minutes. The skin was then dried before measurement to remove any solution. It was verified that the deposit of an electrolytic solution free from antioxidants did not modify the shape of the current-potential curve obtained on the *stratum corneum* surface. On the reverse, when the aqueous solution contained one of the antioxidant species, the voltammogram recorded on the treated area exhibited a higher anodic current than that obtained on the untreated area, as illustrated in Fig. 4. The global antioxidant properties of the skin were therefore enhanced and the surface concentration of the corresponding antioxidant specie was increased. Furthermore, the potential range where the curve increases significantly corresponds to the $E_{1/2}$ previously recorded in the solution containing the corresponding antioxidant, i.e. between 0.2 and 0.6 V/SCE for ascorbic acid, between 0.8 and 1.0 V/SCE for uric acid and around 1.2 V/SCE for glutathione. In this later case, a high current peak at around 0.9 V/SCE was also recorded, indicating an increase of other antioxidants like ascorbic acid. This is not surprising since glutathione is a strong reductive species known to regenerate ascorbic or uric acids [23]. All these results tend to prove that the present non invasive electrochemical method allows the determination of three among the major cutaneous hydrophilic antioxidant species.

3.4. Preclinical applications

3.4.1. Evolution of amperometric signal with time

The evolution of the global antioxidant capacity of the skin with time cannot be studied with invasive methods. For the first time, our non invasive electrochemical method allowed kinetic studies. A preclinical study was performed involving nine volunteer subjects, five men and four women between 20 and 25 years old and with a skin phototype II. Successive cyclic voltammograms were performed every 15 minutes over seven hours using a new platinum microelectrode for each measurement. Fig. 5 show the typical curve giving the evolution of the anodic current recorded at 0.9 V/SCE as a function of time. A sinusoidal evolution was observed for all volunteers. It should be noted that current values as well as the amplitude and the period of the vari-

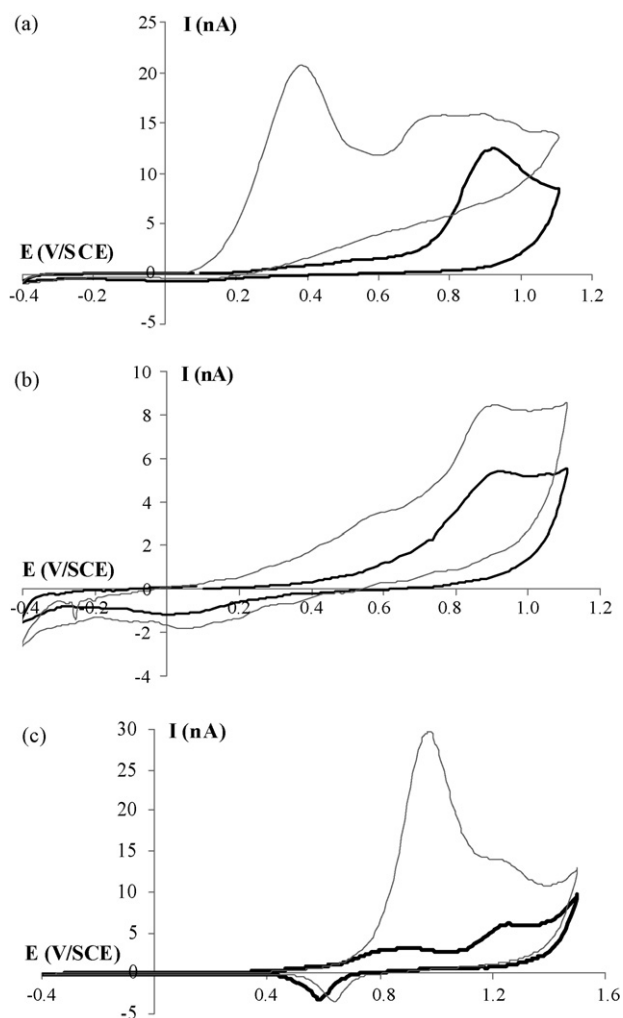


Fig. 4. Cyclic voltammograms obtained directly on a normal skin area (—) and on a skin area previously been in contact with solutions (---) containing (a) ascorbic acid, (b) uric acid or (c) glutathione (0.1 mol L^{-1} , pH 5). Working microelectrode: (a–b) platinum electrode; (c) gold electrode. Potential scan rate: 50 mV s^{-1} .

ations were different for each subject. Mean standard deviation of the amperometric response is indicated for each experimental point; it was lower than the amplitude of the observed variation. So the variation of the anodic current was due to a variation of the redox properties of the skin and was not an artifact of the measurements. The method therefore seems to be suitable to follow the modification of antioxidant levels with time.

The *stratum corneum* can be compared to a porous membrane responsible for the diffusion control. Oxygen diffusing through this barrier reacts with reduced species acting as antioxidant; this one can be further regenerated by another one. This mechanism induces successive oxidoreduction reactions consuming and regenerating antioxidant species. It can be at the origin of the previous electrochemical results.

3.4.2. Evolution of cutaneous antioxidant capacities during a treatment with ascorbic acid

The effect of a topic treatment on the electrochemical response was also studied. A preclinical study was performed during 16 days. Six volunteer subjects were involved, a man and five women between 20 and 35 years old and with a skin phototype II. A dermocosmetic cream (Active C by La Roche-Posay) containing ascorbic acid was applied daily on the inside of the forearm. Cyclic voltammograms were plotted between -0.4 and 1.2 V/SCE with platinum microelectrodes. The anodic charge between 0 V and 0.6 V/SCE was recorded as indicated in Section 3.3.1. This corresponded mainly to the oxidation of ascorbic acid as mentioned in Section 3.3.2. Measurements were performed the first day and 9, 12 and 16 days after on a treated zone and on an untreated zone. Measurements on both zones were realized simultaneously in order to suppress the evolution of the global antioxidant capacity of the *stratum corneum* with time. Cutaneous removals were also systematically done and the corresponding concentration of ascorbic acid was obtained by chromatography as indicated in Section 2.6. Fig. 6 represents the results obtained with the electrochemical (Fig. 6a) and the chromatographic (Fig. 6b) analytical process. In both

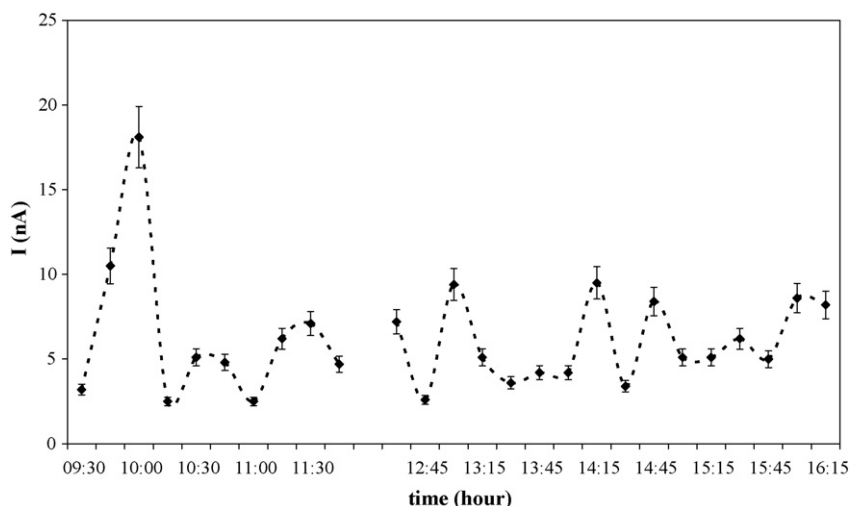


Fig. 5. Evolution of the amperometric current at 0.9 V/SCE recorded with a $50 \mu\text{m}$ diameter platinum microelectrode on forearm skin along seven hours (example of a volunteer). Error bars correspond to the mean measurement accuracy.

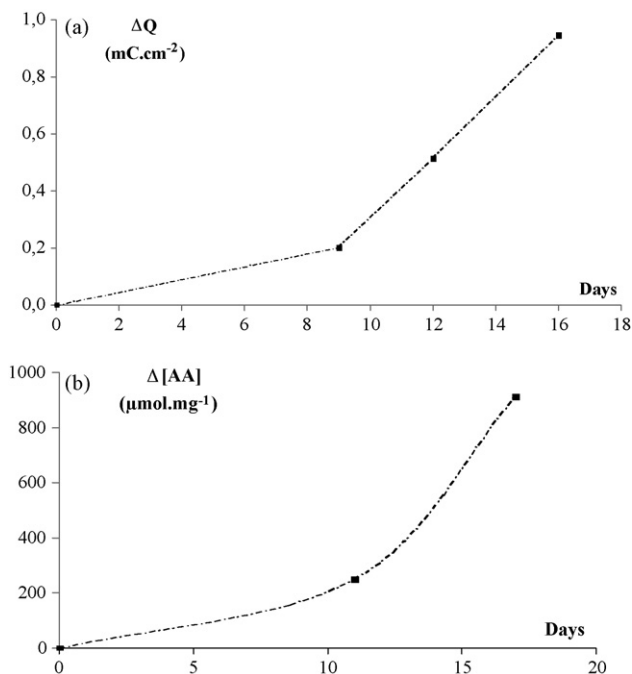


Fig. 6. Evolution with time of the comparative electrochemical results (a) and chromatographic data (b) obtained on a skin area treated with a topic cream containing ascorbic acid and on an untreated skin area. ΔQ corresponds to the difference of anodic charge density recorded between 0 V and 0.6 V/SCE during cyclic voltammograms plotted with platinum microelectrode on both areas. $\Delta[AA]$ corresponds to the difference of concentration of ascorbic acid per mg of proteins obtained by chromatographic analysis. See in the text for protocol details.

cases, the curve indicates the difference of values recorded on the treated area and the untreated one. The two curves showed a similar profile. In both cases no great difference between the treated and the untreated areas was detected during the first week. Both curves then increased, indicating a higher concentration of ascorbic acid on the skin where the cream was applied. All these results show that electrochemical non invasive measurements make it possible to highlight the efficiency of a topic treatment.

3.4.3. Evolution of amperometric signal in eczema areas

In order to test the possibility to realize electrochemical measurements in very dry skin areas and to show the influence of a skin disease on the amperometric response, a preclinical study was performed involving six volunteers suffering from atopic dermatitis. Cyclic voltammograms were simultaneously performed with platinum and gold microelectrodes in a healthy skin area and in an eczema area (Fig. 7). For all subjects, an important decrease of the anodic current was observed in the eczema area. The general shape of the curve obtained in very dry skin area was not modified and no additional resistance was observed. The difference observed between the two areas is thus quite due to a reduction of the global antioxidant capacities of epidermis surface. Thus, these results show that electrochemical methods can be used to underline the modification of antioxidant levels in the case of skin diseases.

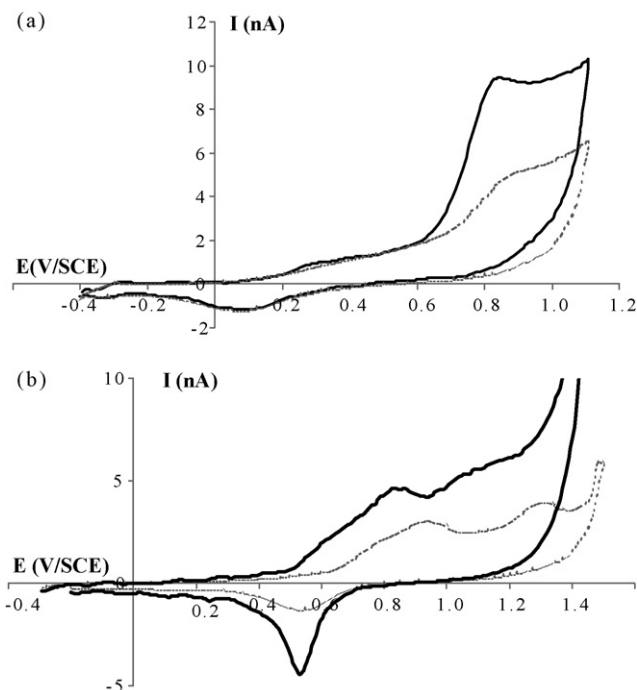


Fig. 7. Cyclic voltammograms obtained directly on a normal skin area (—) and on an eczema area (---) of a volunteer subject suffering from atopic dermatitis. Working microelectrode: (a) platinum electrode; (b) gold electrode. Potential scan rate: 50 mV s^{-1} .

3.4.4. Global antioxidant capacity of skin areas exposed or unexposed to external aggressions

Cutaneous ageing is a complex biological phenomenon consisting of two components: the intrinsic ageing, which is largely genetically determined and the extrinsic ageing caused by environmental exposure, primarily UV light [1,24]. One of our previous study highlighted that the electrochemical method made it possible to show an important decrease of the overall antioxidant skin properties following an UV exposure [17]. In order to take account of all daily external oxidant aggressions, another preclinical study was realized involving nine volunteer subjects between 65 and 70 years old. Cyclic voltammograms were plotted simultaneously in an exposed area, the backside of the hand and in a relatively protected area, the inside of the arm. Gold microelectrodes were used since they allow the detection of sulfur compounds, like cystein or glutathione, particularly sensitive to the oxidation induced by reactive oxygen species [25]. Similarly to results obtained with an eczema area (Section 3.4.3), the anodic charge of the voltammograms strongly decreased when measurement was performed on the exposed area. For all volunteer subjects, the antioxidant capacity of the unexposed area was at least twice higher than that of the exposed area.

4. Conclusion

Microelectrodes allowed direct, rapid, non invasive and precise measurements on skin with high spatiotemporal resolution. The application of relatively simple electrochemical methods, i.e. potentiometry and cyclic voltammetry, allowed the simulta-

neous determination of pH and the antioxidant properties of the cutaneous surface. Furthermore, the selectivity offered by the electrochemical detection made it possible to detect several low molecular weight antioxidant species that prevent from oxidative stress. Finally, the results obtained in real time gave the opportunity to highlight the evolution of the antioxidant properties with time or to follow the effect of a topic treatment. Works are now in progress to obtain quantitative data by correlating all these electrochemical results with the surface concentration of the reduced species.

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