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New concepts for transdermal delivery of oxygen based on catalase biochemical reactions studied by oxygen electrode amperometry

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Abstract

The development of formulation concepts for improved skin tissue oxygenation, including methods for measuring oxygen (O₂) transport across biological barriers, are important research topics with respect to all processes that are affected by the O₂ concentration, such as radiation therapy in oncology treatments, wound healing, and the general health status of skin. In this work we approach this topic by a novel strategy based on the antioxidative enzyme catalase, which is naturally present in the skin organ where it enables conversion of the reactive oxygen species hydrogen peroxide (H₂O₂) into O₂. We introduce various applications of the skin covered oxygen electrode (SCOE) as an in-vitro tool for studies of catalase activity and function. The SCOE is constructed by placing an excised skin membrane directly on an O₂ electrode and the methodology is based on measurements of the electrical current generated by reduction of O₂ as a function of time (i.e. chronoamperometry). The results confirm that a high amount of native catalase is present in the skin organ, even in the outermost stratum corneum (SC) barrier, and we conclude that excised pig skin (irrespective of freeze-thaw treatment) represents a valid model for ex vivo human skin for studying catalase function by the SCOE setup. The activity of native catalase in skin is sufficient to generate considerable amounts of O₂ by conversion from H₂O₂ and proof-of-concept is presented for catalase-based transdermal O₂ delivery from topical formulations containing H₂O₂. In addition, we show that this concept can be further improved by topical application of external catalase on the skin surface, which enables transdermal O₂ delivery from 50 times lower concentrations of H₂O₂. These important results are promising for development of novel topical or transdermal formulations containing low and safe concentrations of H₂O₂ for skin tissue oxygenation. Further, our results indicate that the O₂ production by catalase, derived from topically applied S. epidermidis (a simple model for skin microbiota) is relatively low as compared to the O₂ produced by the catalase naturally present in skin. Still, the catalase activity derived from S. epidermidis is measurable. Taken together, this work illustrates the benefits and versatility of the SCOE as an in vitro skin research tool and introduces new and promising strategies and formulation concepts for transdermal oxygen delivery, and simultaneous detoxification of H₂O₂, based on native or topically applied catalase.

Key words: skin tissue oxygenation; topical and transdermal oxygen delivery; epidermis; stratum corneum; catalase; skin microbiota; hydrogen peroxide; oxygen electrode
1. Introduction

The development of concepts related to improved oxygenation of skin and other tissues, including the development of methods for measuring oxygen (O₂) transport across biological barriers, are important research topics for all processes that are affected by the O₂ concentration. For example, in oncology treatments, involving radiation therapy or photodynamic therapy, the level of O₂ is crucial for suppressed development of tumors after ionizing radiation and generation of reactive oxygen species (ROS) [1, 2]. Other biologically relevant processes, where the O₂ concentration is an important factor, are related to wound healing and the overall health status of the skin barrier. The skin is the only organ, except for the lungs, that is in direct contact with external atmospheric O₂ and it has been shown that the upper skin layers are almost exclusively supplied by external O₂ [3, 4]. Considering this, it is likely that some superficial skin defects may be related to insufficient skin oxygenation from the atmosphere, rather than by a malfunction in the capillary O₂ transport, which has been suggested [3].

The fact that skin is exposed to atmospheric O₂ also means that this organ is highly exposed to oxidative stress from generation of ROS. Therefore, it is perhaps not surprising that the skin organ comprises a robust antioxidative system consisting of both molecular antioxidants and antioxidative enzymes such as catalase, superoxide dismutase, glutathione peroxidase, peroxiredoxin, and heme oxygenase [5]. In particular, catalase is highly expressed in the skin organ and its presence increases towards the O₂ rich atmosphere. In fact, the presence of catalase in skin is nearly one order of magnitude higher in epidermis as compared to the underlying dermis [6]. Further, it should be noted that catalase in skin is present not only in the viable dermis and epidermis, but also in the most superficial part of the skin, the stratum corneum (SC), which is often considered as being a dead tissue [6]. In other words, there is a good correlation between the expression of catalase, as a function of skin depth, and the concentration of O₂, derived from the external atmosphere [3, 6].

The main catalase reaction is conversion of hydrogen peroxide (H₂O₂) into water (H₂O) and O₂ according to H₂O₂ → H₂O + O₂, which may be seen as a detoxification process. In line with this, reduced expression of catalase in skin has been associated to skin diseases, such as vitiligo, and to compensate for this loss and treat some skin disorders, topical application of exogenous and artificial catalase has been proposed [7]. Moreover, recognizing that catalase reaction generates O₂, the application of topical formulations containing H₂O₂ and catalase has been attempted as a solution for topical delivery of O₂ into wounds or ischemic skin tissue [8]. Catalase can also catalyze peroxidase-type reactions by oxidizing suitable hydrogen donors, such as polyphenols or ethanol, with production of acetaldehyde according to H₂O₂ + CH₃CHO → 2H₂O + CH₃CHO [9]. Here, it should be noted that catalase is the only enzyme of the antioxidative system that produce O₂ after exposure to H₂O₂. Further, it is relevant to point out that no O₂ is produced in the case for other substrates, such as alcohols or polyphenols. These facts are taken advantage of in this work where we use an electrochemical experimental setup that measures O₂ and is therefore specific towards catalase activity after exposure to H₂O₂.

Taken together, there is a considerable need for monitoring and understanding catalase function in skin to exploiting this enzyme for improved skin health and development of concepts related to enhanced oxygenation of the skin tissue. To approach this topic, it is crucial to have methods for measuring O₂ transport across the skin barrier and how the concentration of O₂ changes in the skin tissue. A substantial knowledge about catalase reactions in the skin organ and transdermal O₂ delivery can be gained by using relevant in-vitro tools, which minimizes the need for human or animal studies. In this work, we demonstrate that the skin covered oxygen electrode (SCOE) is a useful in-vitro tool to monitor the function of catalase in skin. In this
context, it should be mentioned, that utilization of the SCOE setups for studies of transdermal delivery have been introduced by us in 2015 [10, 11]. In 2017 Nocchi et al. illustrated that the SCOE can be used to monitor reactions that involve native epidermal catalase [12]. In this work we extend the use the SCOE setup and introduce several applications of this in vitro tool to characterize transdermal delivery of O₂ from H₂O₂ solutions and show that catalase is present both in SC and in the viable epidermis where it can oxygenate the skin tissue. In addition, we show that topically applied catalase, including catalase derived from Staphylococcus (S.) epidermidis (as a primitive model of skin microbiota) can be used as a source for increased skin oxygenation.

2. Materials and methods

2.1. Materials

Hydrogen peroxide (H₂O₂, 35 %), phosphate buffer saline (PBS, pH 7.4) in tablets, tannic acid, catalase from bovine liver (2000-5000 units/mg), sodium azide (NaN₃), 3-amino-1,2,4-triazole (3AT), and polyethylenimine were purchased from Sigma-Aldrich (Darmstadt, Germany). Fresh Staphylococcus epidermidis (S. epidermidis) cultures, with colony-forming units of 8x10⁶ cfu/mL, were provided by Biogaia AB (Lund, Sweden). The oxygen electrode consisted of a 5 μm thick Teflon membrane, a 250 μm diameter platinum (Pt) electrode melted in glass, and an internal Ag/AgCl reference electrode; purchased from Optronika UAB (Vilnius, Lithuania). All solutions were prepared by using ultrapure water with a resistivity of 18.2 Ωcm.

2.2. Preparation of split-thickness skin and stratum corneum (SC) membranes

Fresh pig ears were obtained from a local abattoir and stored at -80 °C until use. To prepare skin membranes the ears were thawed and cleaned under flow of cold tap water. Cleaned ears were cut into strips with a scalpel and shaved. Pieces of approximately 500 μm thick skin membranes were sliced with a dermatome. The resulting skin stripes were punched out to make circular membranes with 16 mm diameter. These membranes were kept frozen (-20 °C) until use, usually not longer than four weeks. Before use, the membrane was thawed by placing them on a filter paper, soaked with PBS, and kept for 1-2 hours at room temperature (22°C).

Human breast skin, which is regarded as discarded tissue, was obtained from an anonymous female donor of Caucasian origin and provided by Medibiome AB (no ethical approval is necessary for unidentified residual tissue). Freshly obtained human skin were used within three days and stored in the fridge soaked in saline (0.9 % NaCl). The human skin samples were about 3 mm thick and included the adipose tissue, which is not optimal for the present SCOE setup. Normally, the adipose tissue is easily removed by using a dermatome or scalpel. However, due to the relatively small area of the human skin samples this was a challenging task. Therefore, human skin was only investigated in the form of SC membranes, which are conveniently prepared by trypsin treatment.

SC membranes (approximately 10-30 μm thick) from pig and human skin were prepared by soaking full thickness or split-thickness skin membranes in 0.1 % trypsin solution in PBS for 24h at 4°C. After that, the SC layer was easily removed by forceps, washed with PBS and cleaned with cotton tipped applicators from residual tissue. The SC membranes were immediately mounted on oxygen electrodes for SCOE measurements.

With regards to enzyme viability and storage protocol, in general, it is expected that the enzyme activity is better preserved inside intact tissue samples, or crude extracts, etc., as compared to purified samples where removal of important matrix components may lead to poorer enzyme activity. Considering that the experiments in this work were conducted with relatively intact skin tissue samples, in combination with the fact that relatively high catalase activity was observed...
in these experiments, we conclude that the viability, in terms of catalase activity, was fully satisfactory in all samples studied herein (even after freeze-thaw treatment). Further, there are studies in support of this conclusion where similar storage conditions as used here were investigated [13, 14].

2.3. Preparation of skin covered oxygen electrode (SCOE)

The SCOE was prepared as described previously [12]. Briefly, the surface of the Pt cathode of the oxygen electrode was polished using an alumina suspension (1 μm alumina particles, Buehler, Lake Bluff, IL) and rinsed with deionized water. The body of the electrode was filled with saturated KCl solution and covered with a 5 μm Teflon membrane. Next, the electrode was immersed into a solution of tannic acid (1 mg/mL in PBS) for 3 minutes and then washed in PBS to remove any loosely bound catalase. Initially, it was concluded that the catalase-doped SCOE was significantly more sensitive to exposure to H₂O₂ as compared to normal (untreated) SCOE. To optimize the protocol, we investigated if the results were improved by repeating the described protocol several times. For this, the catalase adsorption steps were repeated so that the total times of immersion into the catalase solution were 3, 6, 9, or 12. From these experiments it was concluded that 3 times was sufficient to achieve a significant increase in the sensitivity, in terms of O₂ production after H₂O₂ exposure, as compared to the normal SCOE. However, the results improved in terms of reproducibility when the catalase adsorption protocol was repeated at least 6 times, without any further benefits of 9 and 12 repeats. Thus, the described protocol was repeated 6 times (at least).

2.4. Topical catalal treatment of the skin covered oxygen electrode (SCOE)

In order to attach catalase on the outer skin surface, the tip of the assembled SCOE was first immersed into a solution of tannic acid (1 mg/mL in PBS) for 3 minutes. Tannic acid is a recognized agent for agglutination and mediator for increased protein adsorption [15, 16]. In other words, the reason for this procedure was to increase the adsorption of catalase on the surface of the skin membrane. Next, the SCOE was washed in PBS for 1 minute, followed by immersion into a catalase solution (10 mg/mL in PBS) for 3 minutes. Finally, the SCOE, with topically adsorbed catalase, was washed in PBS to minimize the presence of any loosely bound catalase. Initially, it was concluded that the catalase-doped SCOE was significantly more sensitive to exposure to H₂O₂ as compared to normal (untreated) SCOE. To optimize the protocol, we investigated if the results were improved by repeating the described protocol several times. For this, the catalase adsorption steps were repeated so that the total times of immersion into the catalase solution were 3, 6, 9, or 12. From these experiments it was concluded that 3 times was sufficient to achieve a significant increase in the sensitivity, in terms of O₂ production after H₂O₂ exposure, as compared to the normal SCOE. However, the results improved in terms of reproducibility when the catalase adsorption protocol was repeated at least 6 times, without any further benefits of 9 and 12 repeats. Thus, the described protocol was repeated 6 times (at least).

2.5. Topical treatment of the skin covered oxygen electrode (SCOE) with Staphylococcus (S.) epidermidis culture

To investigate if bacteria from a S. epidermidis culture could adsorb on the skin surface of the SCOE, the tip of the SCOE was immersed into a suspension of S. epidermidis at room temperature for 24h. The bacterial suspension was agitated with magnetic stirrer at 50 rpm. Before measurements, the electrode was washed with abundant PBS solution, after which it was placed into the electrochemical cell.

2.6. Immobilization of catalase and Staphylococcus epidermidis on the Teflon membrane of the oxygen electrode

To attach catalase directly on the Teflon membrane of the oxygen electrode, the electrode was immersed into a solution of tannic acid (1 mg/mL in PBS) for 3 minutes and then washed in PBS for 1 minute. As stated above, tannic acid was used to increase the adsorption of catalase on the surface of the Teflon membrane [15, 16]. Next, the electrode was immersed into a catalase solution (10 mg/mL in PBS) for 3 minutes and finally washed in PBS to remove any loosely bound catalase.

To evaluate the catalase activity in S. epidermidis by the oxygen electrode, the bacteria were attached to the Teflon membrane. For this, the oxygen electrode was immersed into a polyethylenimine solution (1 mg/mL in water) for 3 minutes, followed by washing with water.
for 1 minute. Finally, the electrode was immersed into a suspension of *S. epidermidis* for 3 minutes. The positively charged polyethylenimine is a recognized attachment factor for various cell lines [17] and thus used here to increase the immobilization of the net negatively surface charged *S. epidermidis*.

### 2.7. Amperometric monitoring of catalase reactions using oxygen electrode

The different types of electrodes, i.e. the SCOE and the oxygen electrode modified with either catalase or *S. epidermidis*, were immersed into an electrochemical cell filled with 10 mL PBS (pH 7.4). The current of the electrode was recorded by using a CompactStat potentiostat from IVIUM Technologies (Eindhoven, The Netherlands). The oxygen electrode was connected to the potentiostat in a two-electrode configuration and the amperometric measurement was conducted by applying -0.7 V vs Ag/AgCl/KCl (sat) on a Pt cathode of the oxygen electrode. After a baseline current was established, a defined amount of H$_2$O$_2$ was pipetted into the electrochemical cell to obtain a known concentration. In the presence of active catalase, the reduction current of the oxygen electrode increased due to O$_2$ generation (see Eq. 1). This is true for active catalase either in the form of native catalase inside the skin membrane, externally adsorbed catalase, or catalase derived from adsorbed *S. epidermidis* at the outer skin surface.

In all experiments, the solution surrounding the oxygen electrode was continuously mixed with a magnetic stirrer at 250 rpm and all measurements were conducted at room temperature (22°C).

### 3. Results and discussion

The general aim of this work was to investigate O$_2$ generation by the enzyme catalase by *in vitro* measurements with a skin covered oxygen electrode (SCOE). The general setup of the SCOE and working principle is illustrated in Fig. 1. Fig. 1A shows the construction of the oxygen electrode with an excised skin membrane mounted on top of the Teflon membrane and sealed by an O-ring. A proof-of-concept is presented in Fig. 1B where raw data from a chronoamperometric measurement of the following four experimental conditions is investigated:

I. SCOE in neat PBS without H$_2$O$_2$ ([O$_2$] = 0.26 mM, no N$_2$ bubbling)

II. SCOE in neat PBS without H$_2$O$_2$ and with N$_2$ bubbling ([O$_2$] ≈ 0 mM)

III. SCOE in PBS with 0.5 mM H$_2$O$_2$ and with N$_2$ bubbling (i.e. O$_2$ production only according to Eq. 1)

IV. SCOE in PBS with 0.5 mM H$_2$O$_2$ (no N$_2$ bubbling, i.e. [O$_2$] = 0.26 mM plus O$_2$ production according to Eq. 1)

These four experimental conditions are schematically illustrated in Fig. 1D (I and II), E (III), and F (IV), together with the particular mechanism of O$_2$ generation in each case. In Fig. 1C, the O$_2$ concentration corresponding to the raw data in Fig. 1B is presented. To enable conversion from current into O$_2$ concentration we calibrate each individual SCOE setup by first recording a stable baseline (I) in PBS buffer with known O$_2$ concentration (0.26 mM or 8.3 mg/L at T=22 °C and 1 atmosphere). By this one-point calibration we avoid the variability of individual SCOE setups, which is mainly due to the combined biological variance of O$_2$ permeability and activity of the native catalase in individual skin membranes. Returning to Fig. 1C, the signal corresponding to condition (II) is obtained by bubbling N$_2$ gas through the PBS solution to eliminate dissolved O$_2$. Then, H$_2$O$_2$ is added to generate a defined concentration of 0.5 mM in the PBS solution (III), which clearly results in an increase of the O$_2$ concentration, corresponding to around 0.02 mM, due to conversion of H$_2$O$_2$ into O$_2$ by catalase. Finally, in the last case (IV), the N$_2$ bubbling is turned off and the O$_2$ concentration comes back to the baseline level. In fact, the final O$_2$ concentration is 0.28 mM, which is in perfect agreement with the combined contributions of dissolved O$_2$ in PBS (0.26 mM), in addition to the O$_2$ that was generated from H$_2$O$_2$ by catalase (0.02 mM). For simplicity, all further measurements were performed without N$_2$ bubbling.
Figure 1. (A) Schematic illustration of the skin covered oxygen electrode (SCOE) and its working principle under different experimental conditions. (B) The change in O₂ concentration is registered by a change in the cathodic current. Upon immersion, between approximately 0-10 min, the O₂ concentration is 0.26 mM in PBS solution of the electrochemical measuring compartment. At around 10 min, N₂ is bubbled through the solution, which effectively minimizes the reducing current. Next, around 25 min, 0.5 mM H₂O₂ is added to the solution, which results in an increase of reducing current in proportion to the generated O₂. Finally, after about 40 min the N₂ bubbling is turned off and the signal returns to a level slightly below the baseline current due to the extra O₂ generated by catalase from the added H₂O₂. (C) The corresponding O₂ concentration from the experimental data in (B). (D, E, and F) Schematic representations of the mechanism(s) responsible for the measured O₂. Case I: baseline current corresponding to PBS saturated with O₂. II: minimal baseline current due to N₂ bubbling. III: minimal baseline current due to N₂ bubbling and O₂ produced by catalase from H₂O₂. IV: baseline current corresponding to PBS saturated with O₂, plus O₂ produced by catalase from H₂O₂.

3.1. Activity of native catalase in epidermis and stratum corneum (SC) membranes

Based on the proof-of-concept presented in Fig. 1, we continue this work by illustrating the versatility of the SCOE setup for investigating the function of native catalase residing in excised skin membranes in vitro. For this, the oxygen electrode was covered with either pig split-thickness skin membranes, pig SC membranes, or human SC membranes. By included measurements with human skin we aim at illustrating that the SCOE in vitro tool with pig skin, even after freeze-thaw treatment, is a valid model for ex vivo human skin. Representative measurements from these experiments are presented in Fig. 2.
Figure 2. Change in $O_2$ concentration measured with the SCOE after stepwise addition of $H_2O_2$. Representative results from (A) pig split-thickness skin, (B) pig SC, (C) human SC, and (D) compilation of the change of $O_2$ concentration ($\Delta O_2$), normalized by the change of $H_2O_2$ concentration ($\Delta H_2O_2$), from several measurements of the different types of membranes (A=pig split-thickness, B=pig SC, C=human SC). The error bars represent the standard error of the mean. After two additions of $H_2O_2$, NaN$_3$ is added to inhibit the catalase present in the skin/SC membrane, after which the $[O_2]$ value returns to baseline level.

Fig. 2A illustrates how the concentration of $O_2$ is changed after addition of $H_2O_2$ from a measurement with pig split-thickness skin membranes (i.e. the membrane contains SC, epidermis, and parts of dermis). After establishment of a stable baseline, $[H_2O_2]$ is first changed from 0 to 0.5 mM, which results in $\Delta [O_2] \approx 0.05$ mM. Next, $[H_2O_2]$ is increased from 0.5 to 1.5 mM, which results in $\Delta [O_2] \approx 0.10$ mM. In other word, $\Delta [O_2]$ is approximately proportional to $\Delta [H_2O_2]$. These results confirm that $H_2O_2$ penetrates the skin membrane where it is enzymatically converted into $O_2$ by native catalase, after which the $O_2$ is transported the oxygen electrode for detection (see Fig. 1). This conclusion is confirmed by the fact that addition of NaN$_3$, which is a well-known catalase inhibitor, results in a decrease of $[O_2]$ back to the baseline level [12]. It should be noted that in some cases, when a stable baseline or a stable reading after $H_2O_2$ addition was not fully achieved, the value after roughly 30 minutes was approximated as endpoint (e.g. Fig. 2A). In general, about 10-30 minutes is required to obtain a stable baseline, corresponding to $[O_2] \approx 0.26$ mM, and the time variation is most likely due to biological differences between individual skin membranes. In addition, equilibration of the skin membrane after immersion into the buffer solution, is a complex process, which may involve, for example, hydration-induced changes of the molecular properties of the protein and lipid components, swelling of the corneocytes, and ion redistribution between the membrane and the buffer [18-20].
A natural continuation from the studies employing split-thickness membranes is to investigate if catalase is present in an active form in the outermost skin barrier. For this, the electrode was covered with SC membranes, which were separated from the underlying epidermis by trypsin treatment. In these experiments, we included both pig SC (Fig. 2B) and human SC (Fig. 2C) for a more complete characterization and to investigate if the pig skin model is a valid model for human skin ex vivo. In both cases, the responses of the SC covered electrodes to \( \text{H}_2\text{O}_2 \) were, in principle, similar as compared to the response of the electrode covered with split-thickness skin membrane (Fig. 2A), i.e. stepwise changes of \([\text{O}_2]\) after \( \text{H}_2\text{O}_2 \) addition. This proves that catalase is present in an active form, and able to convert \( \text{H}_2\text{O}_2 \) to \( \text{O}_2 \), inside the SC barrier of both pig and human skin. This is an intriguing result considering the rather solid-like environment of the SC, where a majority of the proteins and lipids are in a rigid molecular state [18, 19]; even though the SC membrane is fully hydrated as in the present experiments.

The results in Fig. 2 show that generation of \( \text{O}_2 \) as a response to addition of \( \text{H}_2\text{O}_2 \) is, clearly, more rapid in the case of only SC (Fig. 2B and C), as compared to the split-thickness membrane (Fig. 2A). Similarly, the response to the catalase inhibitor (\( \text{NaN}_3 \)) is also significantly faster in the case of only SC (Fig. 2B and C) as compared to the split-thickness membrane (Fig. 2A). The diffusional pathway from the solution to the oxygen electrode, in the case of only SC, is much shorter (total thickness of SC is about 10-30 µm), as compared to the thicker skin membranes (total thickness about 500 µm). This indicates that the thickness, and perhaps the hydrophilicity of the viable epidermis, in combination, act to decrease the transport of the relatively hydrophobic \( \text{O}_2 \) molecule across the membrane. However, it cannot be excluded that the SC membrane contains macroscopic barrier defects, as a result from the separation of SC from the underlying epidermis, which could make it easier for \( \text{H}_2\text{O}_2 \) to reach catalase in the SC membrane and/or make it easier for the produced \( \text{O}_2 \) to diffuse to the electrode via defective regions of the SC membrane.

The experiments with SC from human skin resulted in significantly higher generation of \( \text{O}_2 \) after addition of identical amounts of \( \text{H}_2\text{O}_2 \), as compared to SC from pig skin (i.e. higher value of \( \Delta[\text{O}_2]/\Delta[\text{H}_2\text{O}_2] \), see Fig. 4D, \( p \)-value = 0.001, 2-tailed t-test with 2-sample unequal variance). In fact, the SC from human skin gave a similar response as compared to pig split-thickness skin membranes, i.e. no statistically significant difference (\( p \)-value = 0.638). This should be compared to the observed difference of the change of \([\text{O}_2]\) from the experiments with pig SC and the pig split-thickness, which is statistically different at a weak significance level (\( p \)-value = 0.018). Taken together, these results indicate that the catalase activity in ex vivo human skin is higher as compared to pig skin. However, it is appropriate to issue certain caveats here; the pig skin samples were exposed to freeze-thaw treatment and originated from ears while the human skin samples were not freeze-thawed and collected from breast. Therefore, the comparison presented in Fig. 2D should be considered as a qualitative proof-of-principle showing that the different SCOE setups, corresponding to the results in Fig. 2A, B, C, all successfully work according to the principle illustrated in Fig. 1. In other words, a key conclusion is that the present results illustrate that excised pig skin, even after freeze-thaw treatment, is a valid in vitro model for human skin ex vivo for studying native skin catalase function. In general, it is well established that pig skin is a relevant model to human skin in terms of anatomy [21], permeability [22-26], and electrical properties [26, 27]. Following these studies, it would be interesting to perform further investigations, for example with pig and human skin samples harvested from the corresponding skin sites and treated with identical protocols.

### 3.2. New strategies for transdermal delivery of oxygen

From the results presented above it can be concluded that the SCOE setup enables studies of the catalase reaction in skin. The reaction, of course, must involve addition of \( \text{H}_2\text{O}_2 \) and subsequent \( \text{O}_2 \) generation. Keeping this in mind, we will now illustrate the versatility of the SCOE setup as an in-vitro tool to study transdermal delivery of \( \text{O}_2 \) from solutions containing \( \text{H}_2\text{O}_2 \) and
to examine if the methodology can be extended to study the catalase activity in topically
attached S. epidermidis as a simple, but relevant, mimic for skin microbiota. In all cases, the O2
is generated from catalase. However, the catalase is either provided topically, as such, or derived
from topically adsorbed microbiota. Finally, we also include experiments where the catalase or
microbiota is immobilized directly on the Teflon membrane of the oxygen electrode, to illustrate
the concept of catalase-based O2 delivery in a clear and simple model system.

3.2.1. Improved transdermal delivery of O2 from topically applied catalase

It should be noted that exposure of skin to high concentrations of H2O2 may cause severe skin
burns and blistering, which should be kept in mind at the outset for developing safe formulations
containing H2O2 for the delivery of O2. Here, we assessed the feasibility of skin tissue
oxygenation from low concentrations of H2O2 by comparing two different SCOE designs. The first
one consisted of the basic setup with a split-thickness pig membrane, while the second one
included topically attached catalase on the surface of the skin membrane; except for this, the
SCOE setups were identical. To successfully deposit catalase on the skin membrane, the SCOE
was repeatedly immersed into a solution of catalase, 6 times in total (at least), as described in
Materials and Methods. Typical results from these are presented in Fig. 3A and Fig. 3B,
respectively.

**Figure 3.** Change in O2 concentration measured with the SCOE after repeated addition of H2O2
with (A) pig split-thickness skin and with (B) catalase immobilized on the surface of pig split-
thickness skin membrane (treated 6 times in catalase solution). The time of the H2O2 addition,
and the resulting concentrations, are indicated by arrows. Note that the Δ[H2O2] is 50 times
lower in (B), as compared to (A).

In general, when comparing the results in Fig. 3, it is immediately clear that the SCOE with
immobilized catalase on the skin surface responds to significantly lower concentrations of H2O2,
as compared to the basic SCOE design. The basic SCOE, employing pig split-thickness skin,
requires concentrations of approximately 0.5 mM H2O2 for adequate measurements, while no
notable response is achieved for H2O2 concentrations in the range of 0.01-0.04 mM. In other
words, the increase in O2 concentration shown in Fig. 3B is primarily due to O2 produced by
topically applied catalase at the skin surface; and subsequent diffusion of O2 from the skin
surface across of the membrane to the electrode. This is different as compared to the basic SCOE
setup, where H2O2 diffuses into the skin membrane to the site of the native catalase where it is
converted into O2, which then diffuses to the electrode surface. The Δ[H2O2] is 50 times higher
in the experiment with the normal SCOE (Fig. 3A), as compared to the catalase-doped SCOE (Fig.
3B), at the same time as the corresponding values of Δ[O2] only differs by a factor of 5; i.e.
0.10±0.01 and 0.020±0.005, respectively. This implies that the limiting factor in these
experiments is the flux of H2O2 into the skin membrane to the site of catalase, while the
conversion into O2 and the subsequent flux of O2 are relatively fast processes. Thus, in the case
of topically applied catalase, O2 is converted at the surface of the skin, generating a significant
transdermal flux of \( O_2 \) across the skin tissue to the electrode. This is a striking finding that illustrates the potential of combining low and safe concentrations of \( H_2O_2 \) in topical formulations together with topically applied catalase for transdermal delivery of \( O_2 \).

3.2.2. Inhibition of native skin catalase to enable detection of oxygen derived from catalase in *Staphylococcus epidermidis*

When considering enzymes of the antioxidative system of skin in general, including the contribution from native catalase, one should not ascribe all antioxidative activity to the enzymes located inside the skin organ. On the contrary, a substantial part of the antioxidant activity could be attributed to external skin microbiota, which therefore could play a relevant role for maintaining the redox homeostasis of the skin organ. This hypothesis is supported by a recent study demonstrating that *Propionibacterium acnes* on skin produce the antioxidant enzyme radical oxygenase, which thus increases the antioxidant capacity of the skin [28]. To approach this topic, we investigated if the SCOE *in-vitro* setup could be adopted to detect catalase on skin membrane derived from microbiota. In particular, we wanted to investigate if the catalase activity derived from skin microbiota can produce sufficient amounts of \( O_2 \) to be detected by the SCOE, in a similar manner as demonstrated above for native skin catalase and topically applied catalase. For this, we selected *S. epidermidis*, which is a main component of the commensal skin microbiota [29], as a simple model for skin microbiota. In brief, the basic SCOE was immersed in a culture of *S. epidermidis* for 24h, after which it was thoroughly washed before measurements. Initial measurements indicated that the catalase activity from external *S. epidermidis* was relatively low, but detectable. To achieve better sensitivity, and to scrutinize between \( O_2 \) generated by *S. epidermidis* or by native skin catalase, it was decided to irreversibly inhibit the native skin catalase. The fact that the SCOE, after inhibition with NaN3 and thorough washing in fresh PBS, continued to give a considerable response after addition of \( H_2O_2 \) allowed us to conclude that NaN3 is a reversible inhibitor of catalase. Therefore, instead of using NaN3, we used 3AT, which has been reported to be an irreversible inhibitor of catalase [6]. In short, the SCOE was kept in \( H_2O_2 \) and a solution containing 40 mM 3AT for approximately 3h. To evaluate this concept, we performed the following experiments. First, the SCOE was immersed in PBS for about 1h to reach a stable baseline, after which \( H_2O_2 \) was added to obtain a concentration of 1 mM, followed by inhibition with 3AT (curve 1 in Fig. 4A). Next, the SCOE was rinsed in PBS for 10 minutes and immediately exposed to 1 mM \( H_2O_2 \) again (curve 2 in Fig. 4A). After this, the SCOE was kept in either PBS for 24h (curve 3a in Fig. 4A) or in a suspension of *S. epidermidis* for 24h (curve 3b in Fig. 4A), after which the SCOE was evaluated once more by addition of \( H_2O_2 \).

![Figure 4](image_url). 

**Figure 4.** \( O_2 \) production by catalase derived from topically applied *S. epidermidis*. The experimental protocol is illustrated in (A), while the results from several experiments (n=6-3) are summarized in (B) with error bars representing the standard error of the mean. In (A), the basic SCOE, with pig split-thickness skin, was first exposed to 1.0 mM \( H_2O_2 \), to ensure that the...
SCOE setup functioned normally, followed by inhibition with 3AT for approximately 3h (protocol 1: 3AT inhibition). Next, the catalase-inhibited SCOE was rinsed in PBS, followed by repeated exposure in 1.0 mM H$_2$O$_2$ (protocol 2: 10 min in PBS). In the following step, three replicates were treated in neat PBS buffer for 24h (protocol 3a: 24h in PBS), whereas three replicates were treated in a suspension of microbiota culture for 24h (protocol 3b: 24h in S. epidermidis). After these treatments (3a and 3b), the electrodes were again exposed to 1.0 mM H$_2$O$_2$. In (A), all curves are shifted in time so that the addition of H$_2$O$_2$ to the electrochemical cell occurs at the same point, as indicated by the arrow. It is not possible to conclude that the observed increase in O$_2$ generation, after treating the catalase-inhibited SCOE in the S. epidermidis culture, in fact is higher as compared to the non-microbiota treated catalase-inhibited SCOE (p-level 0.343).

In summary, the results in Fig. 4 show that 3AT significantly suppresses the O$_2$ production and that catalase remains inhibited despite of washing the skin membrane in PBS for 10 min or 24h (curve 2 and 3a, Fig. 4A). These results conclude that catalase inhibition by 3AT can be considered as irreversible, in contrast to inhibition with NaN$_3$. Still, it should be pointed out that there is a minor residual O$_2$ generation even after 3AT inhibition (approximately 10%). In fact, a number of experimental efforts, such as repeated conditioning of the SCOE in H$_2$O$_2$/3AT solution, were performed to completely remove this small trace of O$_2$ production without any success. This is perhaps a surprising observation considering that catalase is the only enzyme that generates O$_2$ from the substrate H$_2$O$_2$. However, it is possible that an unidentified catalase-like (i.e. O$_2$ releasing) enzymatic or biochemical reaction can explain this residual oxygen trace. For example, it has been shown that some peroxidases can generate O$_2$ from the substrate H$_2$O$_2$ via the radical anion superoxide O$_2^-$ [5, 30]. Nonetheless, the residual trace of O$_2$ that remained after 3AT inhibition was accepted as it still allowed for evaluation of the amount of O$_2$ produced by catalase originating from topical S. epidermidis, which is shown by curve 3b in Fig. 4A. In particular, a notable difference is observed when comparing the change of [O$_2$] between curves 3a and 3b (Fig. 4A). The described experimental cycle was repeated with multiple individual SCOE setups, with (n=3) and without (n=3) modification with S. epidermidis, and the results are summarized in Fig. 4B. The mean value of case 3b in Fig. 4B is associated with high standard deviation and the difference between the change of [O$_2$] from the different SCOE's, with and without S. epidermidis, is not fully conclusive (3a and 3b in Fig. 4B). In other words, it is not possible to conclude that the observed increase in O$_2$ generation, after treating the catalase-inhibited SCOE in the S. epidermidis culture, in fact is higher as compared to the non-microbiota treated catalase-inhibited SCOE (p-level 0.343). To address this point, we performed similar experiments with only the Teflon membrane as alternative to the more complex situation of catalase-inhibited skin membrane.

3.2.3. Detection of topical catalase and catalase derived from topical Staphylococcus epidermidis

Topical application of catalase has been proposed to compensate for reduced expression of this enzyme in some skin diseases, such as vitiligo [7]. In addition, production of O$_2$ by catalase after application of topical formulations containing H$_2$O$_2$ is a promising concept for topical delivery of O$_2$ into wounds or ischemic skin tissue [8]. If it would be possible to introduce, or promote, commensal skin bacteria containing catalase, with the aim to contribute to the removal of H$_2$O$_2$ from the skin surface and/or to supply O$_2$ to the skin tissue; this would be novel applications for either transdermal O$_2$ delivery or detoxification of H$_2$O$_2$. Therefore, to approach these questions, and in particular to prove that catalase originating from S. epidermidis can provide measurable amounts of O$_2$ from H$_2$O$_2$, we immobilized catalase or S. epidermidis directly on the Teflon membrane of the oxygen electrode (instead of the skin membrane). The results from these experiments are shown in Fig. 5A and B, respectively. The change of [O$_2$] after addition of H$_2$O$_2$ are obvious. In particular, the sensitivity of the electrode with adsorbed catalase is significantly higher as compared to the electrode with topically attached S. epidermidis. Still, it is promising to conclude that topical application of S. epidermidis, which dominates the skin microbiota, can
contribute with catalase activity on the skin. However, the combined results presented in Fig. 4 and 5B illustrate that the procedure of topical application of external microbiota needs to be further optimized to allow for improved transdermal oxygen delivery and/or increased detoxification of H$_2$O$_2$.

![Figure 5](image-url)

**Figure 5.** O$_2$ production by (A) catalase (as such) and (B) catalase derived from *S. epidermidis* immobilized directly on the Teflon membrane of the oxygen electrode. Note that the ∆[H$_2$O$_2$] is 0.01 mM in (A), while the corresponding situation in (B) is ∆[H$_2$O$_2$] = 0.1 and 0.9 mM (i.e. “low” and “high” concentrations).

### 4. Conclusions

A common perception is that skin receives its O$_2$ supply from the internal circulation. However, recent investigations have shown that a significant amount of O$_2$ may enter skin from the external atmospheric O$_2$ and it has been shown that the upper skin layers are almost exclusively supplied by external O$_2$ [3, 4]. Considering this, it is likely that maintenance of the general skin health and successful wound healing are strongly dependent on adequate skin oxygenation [4].

The ability to deliver topical and transdermal O$_2$ to defective skin, such as wounds or ischemia tissue, may allow the clinician to support the metabolically active wounded tissue for improved healing. Several O$_2$ delivery systems have been developed, such as supersaturated O$_2$ emulsions capable of incorporating high levels of O$_2$ [31], topically applied gaseous O$_2$ [32], and sustained transdermal delivery of O$_2$ via silicone tubing channeled subcutaneously [33]. This study reports on a novel proof-of-concept for catalase-based transdermal O$_2$ delivery by conversion of H$_2$O$_2$ as substrate. We introduce several new applications of the skin covered oxygen electrode (SCOE) as an *in-vitro* tool for studies of native or externally applied catalase. The SCOE is made by placing split-thickness skin or stratum corneum (SC) membranes directly on the O$_2$ electrode (Fig. 1). We demonstrate that excised skin membranes have a high amount of native catalase, even in the outermost SC barrier, and conclude that pig skin (irrespective of freeze-thaw treatment) represents a valid model for *ex vivo* human skin for studying catalase function with the SCOE setup (Fig. 2). The activity of native catalase in the skin barrier is high enough to generate a considerable amount of O$_2$ by conversion from H$_2$O$_2$, which enables successful skin tissue oxygenation. We show that this concept can be further improved by topical application of catalase on the skin surface, which enables transdermal O$_2$ delivery from 50 times lower concentrations of H$_2$O$_2$ (Fig. 3). This is an important and promising finding that opens up for development of topical or transdermal formulations containing low and safe concentrations of H$_2$O$_2$ for transdermal O$_2$ delivery.

Taken together, this work illustrate that it is possible to develop novel catalase-based transdermal formulations with the aim to deliver O$_2$ and detoxify H$_2$O$_2$ for accelerated wound healing and strengthening the overall health status of the skin organ. Further, future research efforts should focus on, for example, localization of native catalase in the complex macromolecular matrix of the skin barrier, and how its activity can be regulated, e. g. by...
hydration [20], excipients [34], humectants [35], penetration enhancers [36, 37], UV radiation [38], or various biogenic stressors of the complex neuro-endocrine system [39].

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