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

Aura Rocio Hernández[1,2,3], Marine Boutonnet[1,2], Birgitta Svensson[4], Eile Butler[5], Rolf Lood[6], Kristina Blom[7], Bibiana Vallejo[3], Chris Anderson[8], Johan Engblom[1,2], Tautgirdas Ruzgas[1,2], and Sebastian Björklund[1,2,*]

[1]Department of Biomedical Science, Malmö University, SE-205 06, Malmö, Sweden.

[2]Biofilms - Research Center for Biointerfaces, Malmö University, SE-205 06, Malmö, Sweden

[3]Department of Pharmacy, Universidad Nacional de Colombia, Bogota 1101, Colombia.

[4]Bioglan AB, SE-202 13, Malmö, Sweden

[5]Biogaia AB, SE-223 62, Lund, Sweden

[6]Department of Clinical Science, Lund University, SE-221 84, Lund, Sweden

[7]Medibiome AB, SE-435 43, Pixbo, Sweden

[8]Department of Clinical and Experimental Medicine, Linköping University, SE-581 83 Linköping, Sweden

*Corresponding author: Sebastian.bjorklund@mau.se

Abstract

The development of formulation concepts for improved skin tissue oxygenation, including methods for measuring oxygen (O_2) transport across biological barriers, are important research topics with respect to all processes that are affected by the O_2 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 (H_2O_2) into O_2. 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 O_2 electrode and the methodology is based on measurements of the electrical current generated by reduction of O_2 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 O_2 by conversion from H_2O_2 and proof-of-concept is presented for catalase-based transdermal O_2 delivery from topical formulations containing H_2O_2. In addition, we show that this concept can be further improved by topical application of external catalase on the skin surface, which enables transdermal O_2 delivery from 50 times lower concentrations of H_2O_2. These important results are promising for development of novel topical or transdermal formulations containing low and safe concentrations of H_2O_2 for skin tissue oxygenation. Further, our results indicate that the O_2 production by catalase, derived from topically applied *S. epidermidis* (a simple model for skin microbiota) is relatively low as compared to the O_2 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 H_2O_2, 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 (O_2) transport across biological barriers, are important research topics for all processes that are affected by the O_2 concentration. For example, in oncology treatments, involving radiation therapy or photodynamic therapy, the level of O_2 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 O_2 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 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 some superficial skin defects may be related to insufficient skin oxygenation from the atmosphere, rather than by a malfunction in the capillary O_2 transport, which has been suggested [3].

The fact that skin is exposed to atmospheric O_2 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 O_2 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 O_2, derived from the external atmosphere [3, 6].

The main catalase reaction is conversion of hydrogen peroxide (H_2O_2) into water (H_2O) and O_2 according to $H_2O_2 + H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$, 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 O_2, the application of topical formulations containing H_2O_2 and catalase has been attempted as a solution for topical delivery of O_2 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 $H_2O_2 + CH_3CH_2OH \xrightarrow{Catalase} 2H_2O + CH_3CHO$ [9]. Here, it should be noted that catalase is the only enzyme of the antioxidative system that produce O_2 after exposure to H_2O_2. Further, it is relevant to point out that no O_2 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 O_2 and is therefore specific towards catalase activity after exposure to H_2O_2.

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 O_2 transport across the skin barrier and how the concentration of O_2 changes in the skin tissue. A substantial knowledge about catalase reactions in the skin organ and transdermal O_2 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 O_2 from H_2O_2 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 (H_2O_2, 35 %), phosphate buffer saline (PBS, pH 7.4) in tablets, tannic acid, catalase from bovine liver (2000-5000 units/mg), sodium azide (NaN_3), 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 $8x10^8$ 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 covered with either a split-thickness skin or SC membrane, directly on top of the Teflon membrane, resulting in the SCOE (see Fig. 1A for a schematic representation).

2.4. Topical catalase 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 H_2O_2, 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 O_2 production after H_2O_2 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_2O_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_2O_2 ($[O_2]$ = 0.26 mM, no N_2 bubbling)
II. SCOE in neat PBS without H_2O_2 and with N_2 bubbling ($[O_2] \approx 0$ mM)
III. SCOE in PBS with 0.5 mM H_2O_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_2O_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_2O_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_2O_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_2O_2 by catalase (0.02 mM). For simplicity, all further measurements were performed without N_2 bubbling.

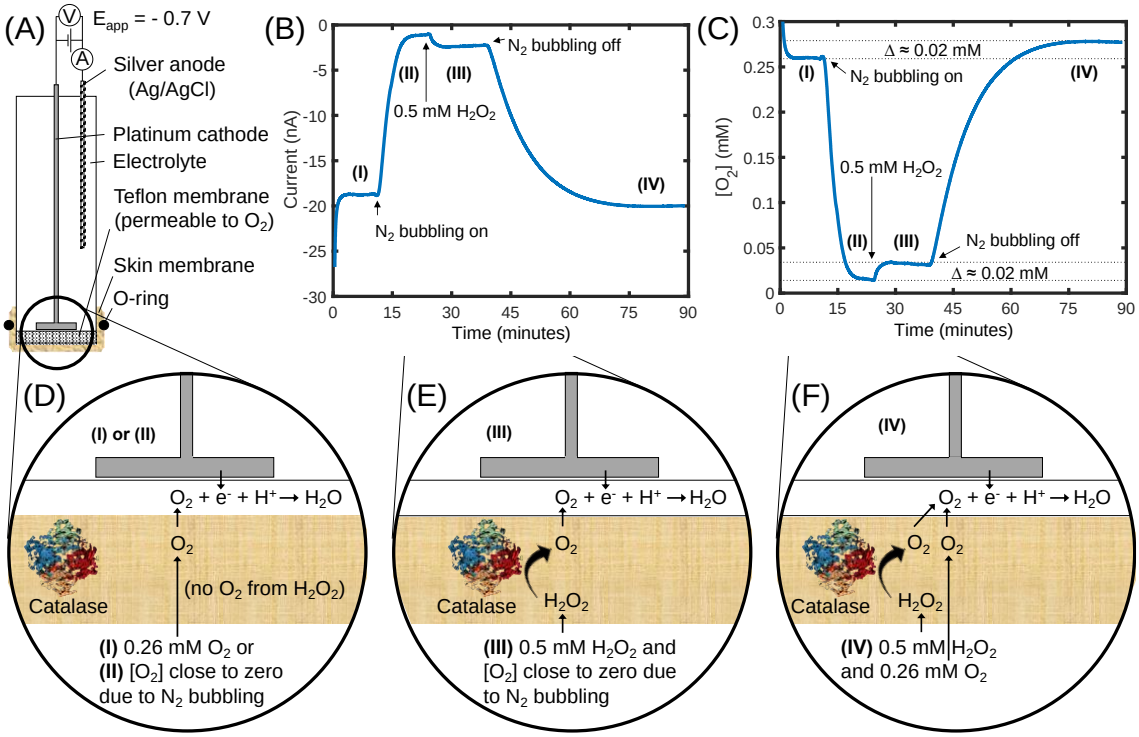

Figure 1. (A) Schematic illustration of the skin covered oxygen electrode (SCOE) and its working principle under different experimental conditions. (B) The change in O_2 concentration is registered by a change in the cathodic current. Upon immersion, between approximately 0-10 min, the O_2 concentration is 0.26 mM in PBS solution of the electrochemical measuring compartment. At around 10 min, N_2 is bubbled through the solution, which effectively minimizes the reducing current. Next, around 25 min, 0.5 mM H_2O_2 is added to the solution, which results in an increase of reducing current in proportion to the generated O_2. Finally, after about 40 min the N_2 bubbling is turned off and the signal returns to a level slightly below the baseline current due to the extra O_2 generated by catalase from the added H_2O_2. (C) The corresponding O_2 concentration from the experimental data in (B). (D, E, and F) Schematic representations of the mechanism(s) responsible for the measured O_2. Case I: baseline current corresponding to PBS saturated with O_2. II: minimal baseline current due to N_2 bubbling. III: minimal baseline current due to N_2 bubbling and O_2 produced by catalase from H_2O_2. IV: baseline current corresponding to PBS saturated with O_2, plus O_2 produced by catalase from H_2O_2.

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.

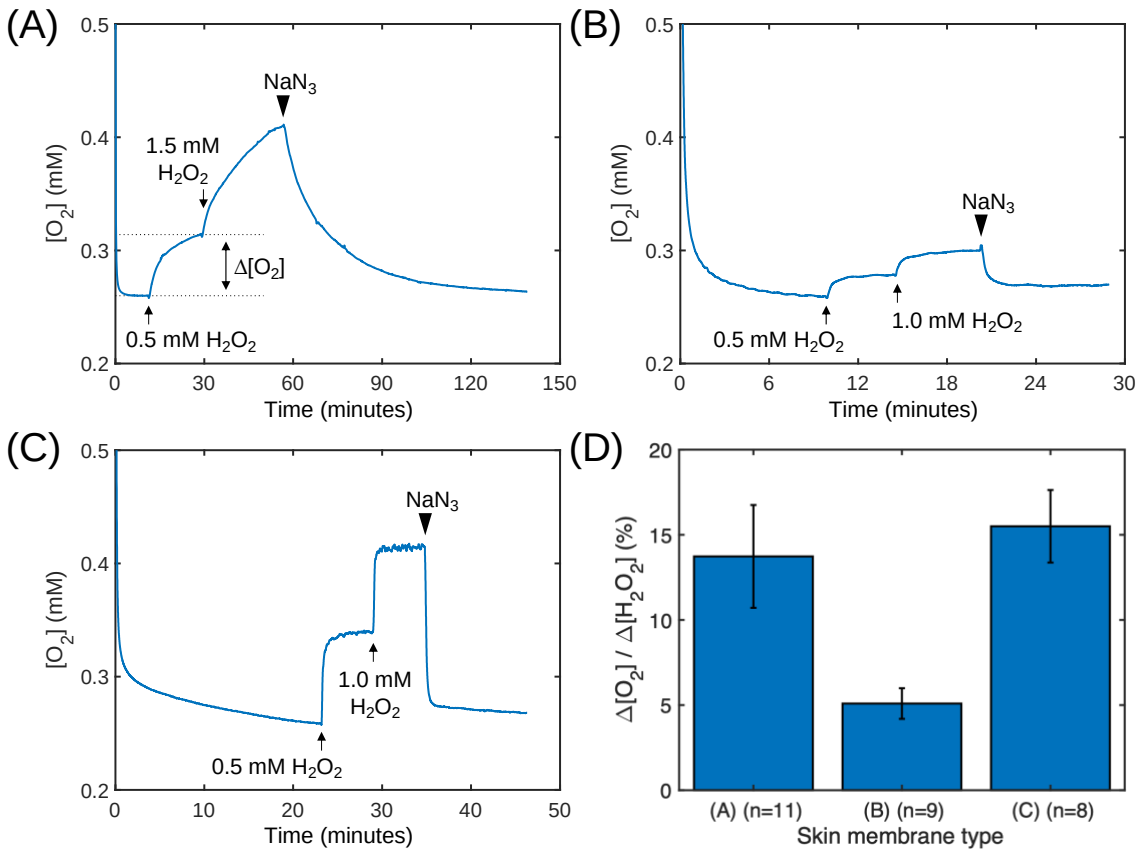

Figure 2. Change in O_2 concentration measured with the SCOE after stepwise addition of H_2O_2. Representative results from (**A**) pig split-thickness skin, (**B**) pig SC, (**C**) human SC, and (**D**) compilation of the change of O_2 concentration (ΔO_2), normalized by the change of H_2O_2 concentration (ΔH_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 $[O_2]$ = 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 H_2O_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 $[O_2]$ after H_2O_2 addition. This proves that catalase is present in an active form, and able to convert H_2O_2 to 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 O_2 as a response to addition of H_2O_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 (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 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 H_2O_2 to reach catalase in the SC membrane and/or make it easier for the produced 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 O_2 after addition of identical amounts of H_2O_2, as compared to SC from pig skin (i.e. higher value of $\Delta[O_2]/\Delta[H_2O_2]$, 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 $[O_2]$ 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 H_2O_2 and subsequent 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 O_2 from solutions containing H_2O_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 O_2 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 O_2 delivery in a clear and simple model system.

3.2.1. Improved transdermal delivery of O_2 from topically applied catalase

It should be noted that exposure of skin to high concentrations of H_2O_2 may cause severe skin burns and blistering, which should be kept in mind at the outset for developing safe formulations containing H_2O_2 for the delivery of O_2. Here, we assessed the feasibility of skin tissue oxygenation from low concentrations of H_2O_2 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.

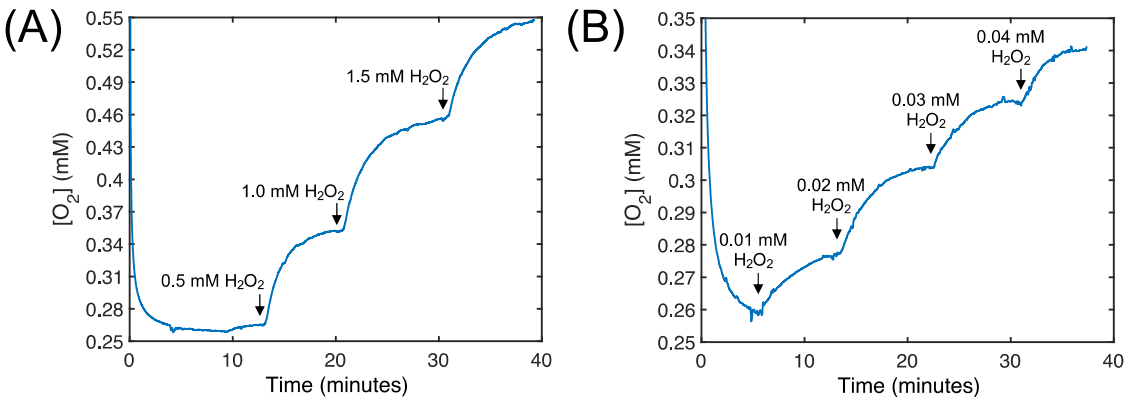

Figure 3. Change in O_2 concentration measured with the SCOE after repeated addition of H_2O_2 with (A) pig split-thickness skin and with (B) catalase immobilized on the surface of pig split-thickness skin membrane (treated 6 times in catalase solution). The time of the H_2O_2 addition, and the resulting concentrations, are indicated by arrows. Note that the $\Delta[H_2O_2]$ 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 H_2O_2, as compared to the basic SCOE design. The basic SCOE, employing pig split-thickness skin, requires concentrations of approximately 0.5 mM H_2O_2 for adequate measurements, while no notable response is achieved for H_2O_2 concentrations in the range of 0.01-0.04 mM. In other words, the increase in O_2 concentration shown in Fig. 3B is primarily due to O_2 produced by topically applied catalase at the skin surface; and subsequent diffusion of O_2 from the skin surface across of the membrane to the electrode. This is different as compared to the basic SCOE setup, where H_2O_2 diffuses into the skin membrane to the site of the native catalase where it is converted into O_2, which then diffuses to the electrode surface. The $\Delta[H_2O_2]$ 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 $\Delta[O_2]$ 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 H_2O_2 into the skin membrane to the site of catalase, while the conversion into O_2 and the subsequent flux of O_2 are relatively fast processes. Thus, in the case of topically applied catalase, O_2 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 NaN_3 and thorough washing in fresh PBS, continued to give a considerable response after addition of H_2O_2 allowed us to conclude that NaN_3 is a reversible inhibitor of catalase. Therefore, instead of using NaN_3, 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.

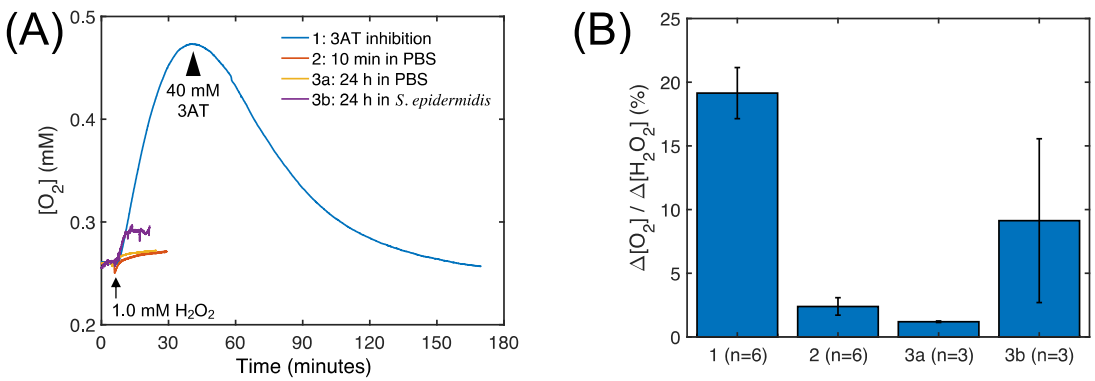

Figure 4. O_2 production by catalase derived from topically applied *S. epidermidis*. The experimental protocol is illustrated in (A), while the results from several experiments (n=6-3) are summarized in (B) with error bars representing the standard error of the mean. In (A), the basic SCOE, with pig 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_2O_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_2O_2. In (A), all curves are shifted in time so that the addition of H_2O_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_2O_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_2O_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_2O_2 via the radical anion superoxide $O_2^{\bullet-}$ [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 SCOEs, 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_2O_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_2O_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_2O_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_2O_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_2O_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_2O_2.

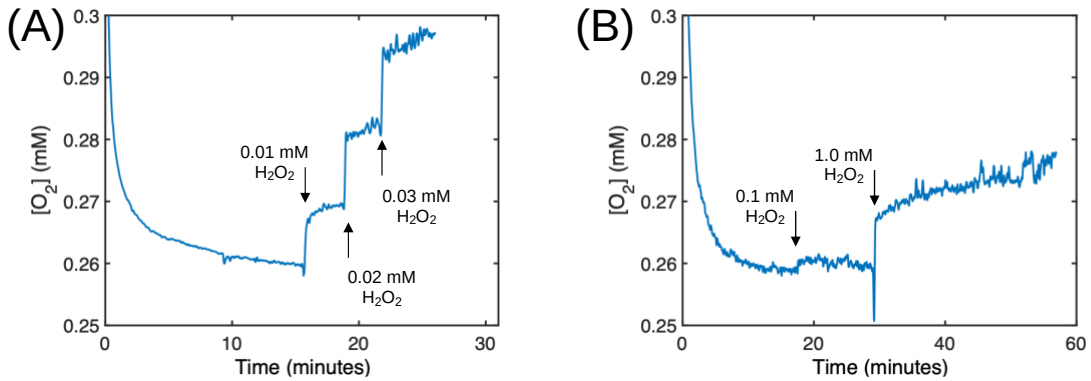

Figure 5. O_2 production by (A) catalase (as such) and (B) catalase derived from *S. epidermidis* immobilized directly on the Teflon membrane of the oxygen electrode. Note that the $\Delta[H_2O_2]$ is 0.01 mM in (A), while the corresponding situation in (B) is $\Delta[H_2O_2]$ = 0.1 and 0.9 mM (i.e. "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_2O_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 is 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_2O_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_2O_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_2O_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_2O_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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