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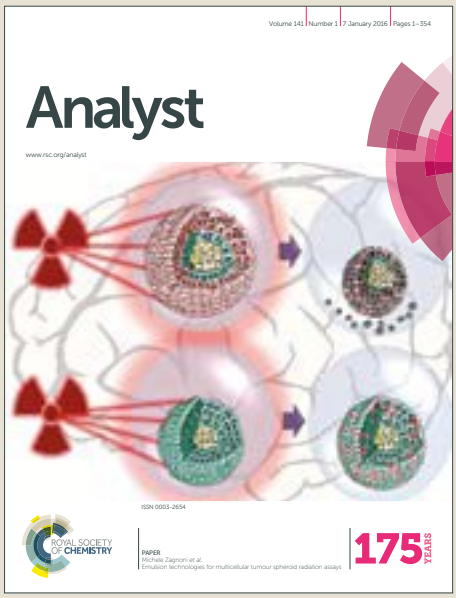

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Microneedle Biosensor for Minimally-Invasive Transdermal Detection of Nerve Agents

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Abstract

A microneedle electrochemical biosensor for the minimal-invasive detection of organophosphate (OP) chemical agents is described. The new sensor relies on the coupling of the effective biocatalytic action of organophosphorus hydrolase (OPH) with a hollow-microneedle modified carbon-paste array electrode transducer, and involves rapid square-wave voltammetric (SWV) measurements of the p-nitrophenol, product of the OPH enzymatic reaction in the presence of the OP substrate. The scanning-potential SWV transduction mode offers additional dimension of selectivity compared to common fixed-potential OPH-amperometric biosensors. The microneedle device offers a highly linear response for methyl paraoxon (MPOx) over the range of 20-180 µM, high selectivity in the presence of excess co-existing ascorbic acid and uric acid and high stability sensor upon exposure to interstitial fluid (ISF). The OPH microneedle sensor was successfully tested *ex-vivo* using mice skin samples exposed to MPOx, demonstrating its promise for minimally-invasive monitoring of OP agents and pesticides and as wearable sensor for detecting threat compounds, in general.

Key Words: Organophosphorus nerve agents, microneedle sensor, Organophosphorus hydrolase, wearable sensor, enzyme electrode

Introduction

Organophosphorus (OP) nerve agents are among the most toxic chemical warfare agents that have been produced, stockpiled and weaponized.[1,2] Multiple studies have shown that these OP neurotoxins affect severely the nervous system, leading to death. Potential exposure to chemical nerve agents due to terrorist and military activity has thus become a serious threat to our soldiers and civilians. Such exposure may lead to internal uptake of the OP compound by two

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routes involving inhalation through the respiratory system, and skin penetration upon dermal exposure. Skin penetration is particularly important for low-volatile agents, such as VX, malathion, and chloropyrifos.[3] Skin adsorption of organophosphorus compounds is also of major concern to farmers exposed to pesticides in agricultural settings, since different pesticides can be absorbed through the layers of the epidermis different penetration rates.[4] This represents a particular hazard among those who mix and apply pesticides. Due to low vapor pressures of many pesticides their respiratory entry appears to be limited compared to the major dermal entry route. Many OP compounds can be readily absorbed through the skin, and can readily cross alveolar and dermal membranes due to their lipophilic structures.[5] After adsorption, OPs are distributed in the whole body, especially in fatty tissues. Due to the high toxicity of OP pesticides and nerve agents, there are urgent needs for rapid, sensitive and reliable sensing tools for their environmental, security and biomedical monitoring. [6,7,] On-body detection of these chemical agents is thus essential for providing rapid warning regarding personal exposure to nerve agents. In particular, rapid detection of their skin penetration is essential to ensure an immediate medical treatment. Biocatalytic sensors, particularly enzymatic probes based on acetylcholine-esterase inhibition or OP sensing using organophosphorus hydrolase (OPH) have played a major role in detecting the presence of OP chemical threats. The OPH has been shown to effectively hydrolyze a range of OP pesticides and chemical warfare agents. Several types of OPH-based biosensors have been described previously, including potentiometric,[8] amperometric,[9,10] and optical[11] ones.

In this article, we report an OPH-based microneedle electrochemical biosensor that can detect OP compounds under the skin rapidly, selectively and directly in a single-step. Microneedle arrays have been developed initially for transdermal drug delivery applications owing to their unique ability to transport molecules into the skin.[12] Such microneedles can be prepared by different fabrication methods to yield a variety of needle geometries, sizes, shapes and materials. Several groups have investigated the use of microneedle arrays as diagnostic tool aimed at transdermal monitoring of different analytes.[13,14]. This activity resulted in microneedle electrochemical biosensors for minimally-invasive monitoring of glucose,[15] lactate,[16] or glutamate,[17] However, there are no reports on developing microneedle sensors for minimally-invasive monitoring of potential security threats or environmental hazards.

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The new OPH-based microneedle transdermal biosensor relies on packing hollow microneedles with carbon paste (CP) electrode transducer, coated with an OPH/Nafion reagent layer. Scanning-potential square-wave voltammetry (SWV) is used for detecting the nitrophenol product of the OPH hydrolysis reaction to offer additional dimension of selectivity compared to early fixed-potential OPH amperometric biosensors. Compared to early fixed-potential biomedical microneedle sensor[15-17] the new potential-scan voltammetric detection imparts higher specificity into microneedle sensing devices. The resulting OPH microneedle biosensor displays direct, rapid, and selective measurement of micromolar concentrations of OP nerve agents along with high stability in interstitial fluid (ISF). The OPH microneedle sensor was tested successfully upon piercing mice skin samples exposed to different levels of the model OP MPOx compound. The microneedle-based biosensing approach offers considerable promise for effective subcutanous electrochemical monitoring of nerve agents and pesticides towards the protection of our soldiers and farmers and can be readily extended to the skin-worn detection of other threat and hazardous compounds.

Experimental

Materials

The enzyme organophosphorus hydrolase (OPH, EC 3.1.8.1 was obtained from Prof J. Marty, (UPVD, Perpignan France). The enzyme activity was 98 IU/mg. This enzyme was first isolated from *Flavobacterium sp*. and cloned in *Escherichia coli*. The enzyme solution was prepared by dissolving 0.7 mg of the OPH lyophilized powder 200 µl of 0.1 M phosphate buffer (PB) (pH 8.0) containing 0.7 mg BSA to enhancing the enzymatic stability. The buffer ingredients KH_2PO_4, K_2HPO_4, Nafion®, mineral oil (d=0.838 g mL^{-1}) ascorbic acid (AA), uric acid (UA), methyl-paraoxon (analytical standard, Lot #SZBD128XV) and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO), graphite powder (crystalline 99% was procured from Alfa Aesar; Ward hill, MA) and were used without further modification. A potentiostat (model 1232, CH Instruments, Austin, TX, USA) connected to a PC, were employed for the SWV. Experiments were carried out in PB 0.1 M pH 8.0 and in artificial interstitial fluid (ISF) prepared according to Fogh-Andersen et al.[18] Ultra-pure deionized water (18.2 MΩ cm) was utilized for all solutions. In

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view of the toxicity of MPOx, all measurements were performed in the fume hood to avoid any harmfull effects.

Instrumentation and Procedure

Electrochemical measurements were carried out using a CH Instruments (Austin, TX) Model CHI 1232A electrochemical analyzer. In these square-wave voltammetric electrochemical investigations, MPOx was added into a 0.1M PB (pH 8.0) solution to obtain the desired concentration. In order to obtain the anodic voltammograms of *p*-nitrophenol enzymatic product, a 30 sec SWV potential scan over 0.3-1.0V was applied (vs. Ag/AgCl). The MPOx solution was static on the microneedle surface throughout the analysis. Photographs of microneedle arrays were captured using an advanced digital camera (Nikon, D7000).

Hollow microneedle array fabrication

The hollow microneedle arrays were prepared similarly to those used in as our early studies.[13,15] The 3x3 hollow microneedle arrays had pyramidal shape with a triangular base. The needles had a height of 1500 µm, and vertical cylindrical holes (425 µm diameter) on the side of their pyramid structure. The substrates for the hollow microneedle arrays (10 mm × 10 mm) had a thickness of 500 µm. The hollow microneedle arrays were prepared using Solid works 3-D computer models. These models were transferred to a Perfactory® SXGA Standard UV rapid prototyping system (EnvisionTEC GmbH, Gladbeck, Germany) for construction by guiding light from a 150 W halogen bulb over a photo curable material, followed by selective polymerization of the exposed material. Acrylate-based polymer (Eshell 200 Envision, TEC GmbH) served as the principal material to fabricate the microneedles since the resin selectively polymerizes under visible light and displays a Young's modulus of elasticity of 3050 ± 90 MPa.[19] Polymerization of the resin involved a 550 mW output power beam (step size = 50 µm) with a zero-degree slope. Following fabrication, the microneedle arrays were washed with isopropanol for removing the unpolymerized material, and subsequently were cured in an Otoflash post-curing system (EnvisionTEC GmbH, Gladbeck, Germany).

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Packing of the microneedles

Most experiments were carried using three of the CP packed microneedles in the array, in which two of them used as working and counter electrodes and the third one was modified using Ag/AgCl ink (towards a reference electrode). Each single electrode had a surface area of 0.002 cm^2. The CP was prepared by using graphite powder (65 wt %) as the conductive material and mineral oil (35 wt %) as the binder. Both constituents were mixed thoroughly together until a homogenous CP was formed. The paste was filled in the hollow microneedles and the excess paste was carefully detached from the surface; the resulting CP surface was smoothed using a wax paper. The back sides of the CP packed needles were connected with stainless steel wires using conductive silver epoxy in order to create electrical contacts. The Ag/AgCl ink in the third microhole was cured at 90 °C for 15 min and served as the reference electrode.

Construction and evaluation of OPH microneedle sensors

The OPH enzyme was immobilized on CP working electrode microneedle transducer using a 1% Nafion layer which prevents its leaking while providing an anti-interference barrier. The OPH solution was prepared by dissolving 0.7 mg of the enzyme lyophilized powder in 200 µl of 0.1 M PB (pH 8.0), containing 0.7 mg BSA, to enhance the enzymatic stability. This solution, was mixed with Nafion (1% w/v in ethanol) at the ratio of 3:7. Subsequently, 4 µL of the polymer/enzyme mixture was drop casted over the working microelectrode resulting in an OPH biofilm and allowed to dry it under ambient condition for 2h.

The stock solutions of MPOx were prepared using acetonitrile. To test the control, 80 µL PB (pH 8.0) was dispensed on to the microneedle array, submerging all 3 electrodes. Different MPOx concentrations (in 0.1M PB) were used to assess the dynamic range and selectivity using SWV measurements over the potential 0.3-1.0V range (vs. Ag/AgCl reference electrode). The artificial ISF was prepared by following the procedure reported by Fogh-Andersen et al.[16]

Skin test using microneedles

The mice skin samples were collected and stored at -20°C prior to use. On the day of experiment, the mice skin was thawed at room temperature and the skin samples were cut into 5.52 cm^2 rectangular pieces. The full-thickness of unclipped skin was used. The skin samples were placed

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in an inverted petri-dish for convenient microneedle accessibility under the fume hood. For *ex-vivo* OP sensing within the skin, three different MPOx concentrations (50, 100 and 150 µM) were prepared in 0.1M PB (pH 8.0) and a100 µL aliquot of each sample was dispensed onto the skin surface. The MPOx was allowed to absorb by the skin samples in fume hood for 20 min. Subsequently, the contaminated skin surface was thoroughly cleaned by wiping with Kimwipe to ensure complete removal of MPOx from the surface. The skin was then pierced with OPH microneedle sensor to facilitate electrochemical monitoring of OP nerve agent within the skin.

Results and Discussion

Initial optimization for OPH microneedle sensor

The detection process at the new microneedle biosensors relies on the anodic detection of the *p*-nitrophenol product of the selective enzymatic reaction of the OP substrate at the OPH/Nafion

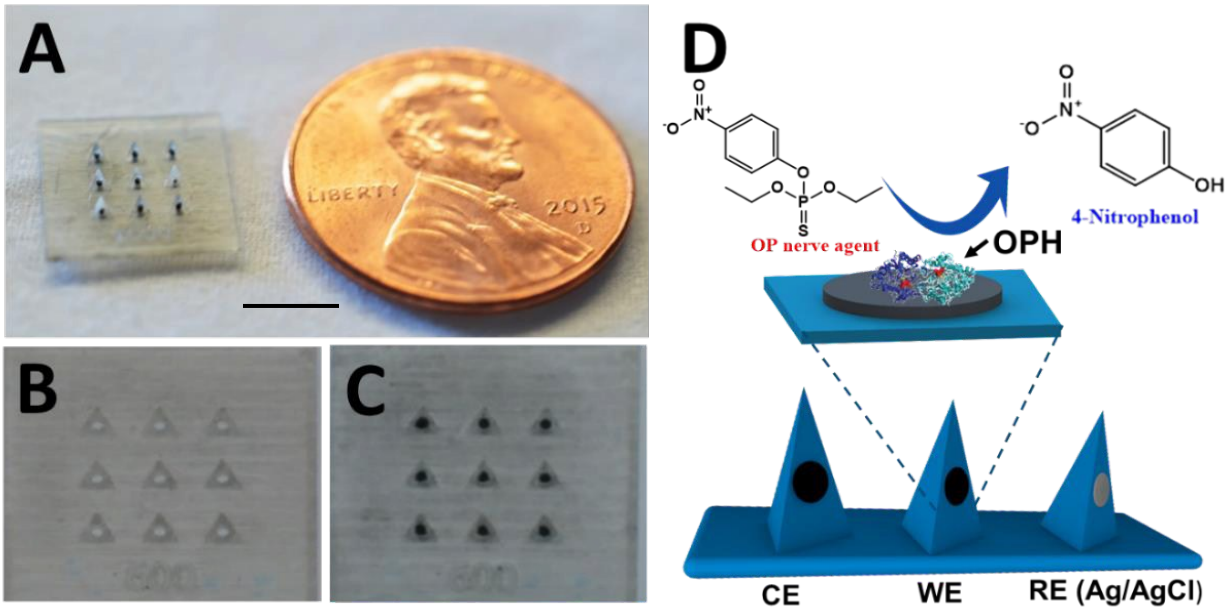

Figure 1: *Schematic of transdermal OPH microneedle electrode sensing. A) Actual picture of hollow microneedle array; scale bar 10 mm. Optical images of the hollow (unpacked) (B) and carbon-paste packed (C) microneedles. D) Schematic representation of electrochemical detection of OP nerve agents using OPH microneedle sensor based on the carbon- and Ag/AgCl-based microneedles.*

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layer coating the surface of the CP transducer. Figure 1 displays actual images of the transdermal microneedle electrode (A-C) and schematic illustration of the biocatalytic reaction at the microneedle working electrode (D). A well-defined pattern of 9 hollow microneedles, with a uniform pyramidal structure and a triangular base, is observed (B). As illustrated in the actual photograph (C), these hollow microneedles have been fully packed with the CP electrode transducer whose surface has been smoothed while removing the excess paste. These images also illustrate that the electrode openings are located on the side of the pyramidal surface. Overall, the design and materials of these microneedle arrays facilitate the penetration/insertion of the microneedle constituents to permeate the *stratum corneum* and access the ISF without their bending, while ensuring minimal damage upon piercing the skin and reproducible response. Such high-aspect ratio microneedles with sharp rigid tips are essential for providing successful and reliable skin penetration.[20] The CP electrode transducer was coated with a biocatalytic/permselective reagent layer. Nafion (1%) was selected as the permselective layer owing to its attractive charge-exclusion properties.[16] Various OPH and Nafion ratios were evaluated with the 3:7 OPH/Nafion ratio offering the optimal response. This composition was used in all subsequent work.

Electrochemical performance of OPH Microneedle sensor

The anodic signal, arising from the oxidation of the p-nitrophenol product of the OPH biocatalytic reaction, is proportional to the level of the hydrolyzed nerve agent.[7,8] The performance of the OPH microneedle sensor towards the detection of MPOx was initially assessed in 0.1M PB (pH 8.0). Unlike early OPH-based biosensors that involved fixed-potential amperometric measurements of the nitrophenol product, [4] the operation of the new microneedle biosensor relies information-rich scanning-potential voltammetric detection. Among the potential voltammetric transduction modes, SWV is most promising as it combines effective discrimination against the charging (background) current, i.e., high sensitivity, with a high speed (2-5 sec scan).[21] Optimal SWV detection conditions were established and included a potential range of 0.3-1.0V, along with an amplitude of 25 mV and a potential step of 4 mV. Figure 2a illustrates square-wave voltammograms recorded at the OPH microneedle biosensor for increasing MPOx concentrations from 20 to 180 µM (20 µM increments; a-i). These voltammograms display a well-defined oxidation peak (at +0.75V) that allows convenient measurements of these micromolar MPOx concentrations. The resulting

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calibration plot of the background-subtracted peak-current vs MPOx concentration (shown in the inset) is highly linear (R^2=0.999). A detection limit of around 4 µM can be estimated based on the signal-to-noise (S/N=3) characteristics of the response to 5 µM MPOx (a). A control experiment, carried out without OPH enzyme in the Nafion reagent layer (shown in Figure 2B), indicates no apparent response to increasing MPOx concentrations, with signals similar to those observed for the blank buffer solution.

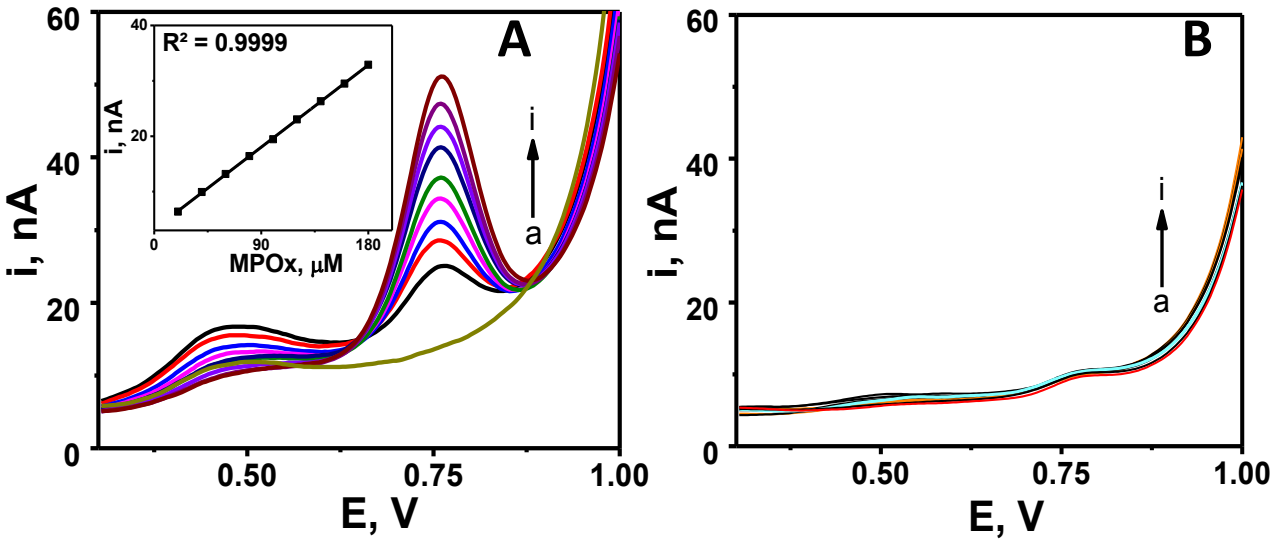

Figure 2: *OPH-based microneedle biosensing of the nerve agent MPOx;* ***A)*** *Square-wave voltammograms for MPOx concentrations from 20 to 180 µM in 20 µM increments (a-i). The inset shows the calibration plot of the background-subtracted peak-current vs MPOx concentration. SWV potential range, 0.3 - 1.0V vs. Ag/AgCl* using *a frequency of 10 Hz, and amplitude of 25 mV.* ***B)*** *Control experiments using the Nafion coating without OPH, over the 20-180 µM MPOx range in 20 µM increments.*

Selectivity of the OPH microneedle sensor

The selective detection of the target OP agent is a prime concern for the development of threat biosensors with minimal false alarms. Since the new OPH microneedle is expected to detect OP nerve agents transdermally (in ISF), the transducer surface must display high selectivity towards the target OP analyte even in the present of large excess co-existing compounds, particularly the easily-oxidizable electroactive ascorbic acid (AA) and uric acid (UA) that commonly affect

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subcutaneous electrochemical measurements.[22] The selectivity of the response was examined in the presence of physiological levels of AA (250 µM) and UA (250 µM). Figure 3 illustrates the voltammetric response of the microneedle sensor to 50µM MPOx in the presence of 250 µM AA and UA. These voltammograms (and corresponding bar diagram) indicate that these excess levels of the co-existing electroactive species have a negligible effect upon the current signal for 50 µM MPOx. Such highly selective response reflects the combined effect of the specific OPH biocatalytic reaction, the anti-interference (charge-exclusion) properties of the polymeric Nafion barrier and of the voltammetric detection mode. The electroactive analytes AA and UA were also tested separately in artificial ISF and displayed no detectable SWV anodic signals (not shown). Such interference-free nerve agent detection further supports the suitability of the OPH microneedle for subcutaneous monitoring in transdermal fluids.

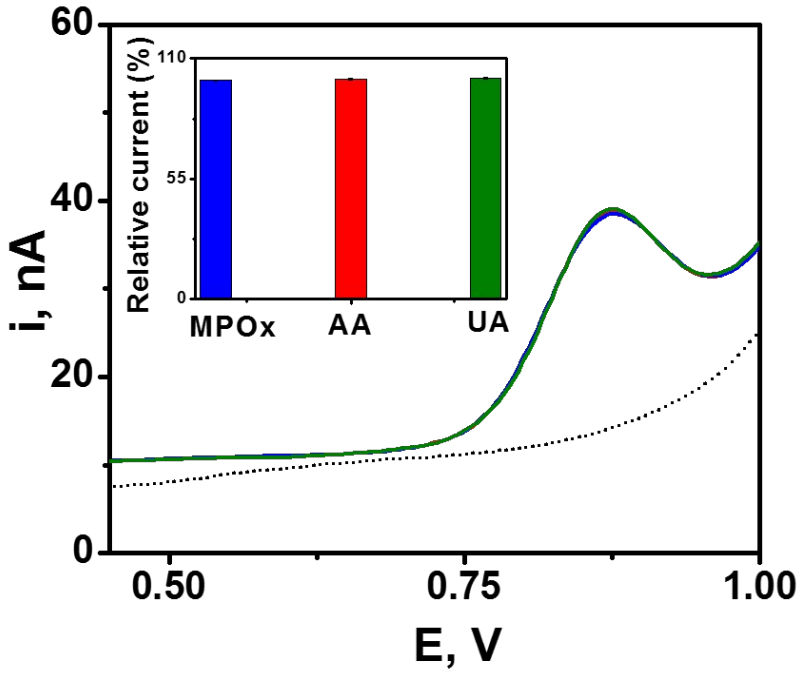

Figure 3: *Selectivity of the OPH microneedle biosensor in artificial ISF towards 50 µM MPOx (blue) in the presence of 250 µM AA (red) and 250 µM for AA (green). The dash curve represents the baseline response of the artificial ISF. Inset represents the relative current signals for 50 µM MPOx. Conditions, as in Fig. 2A.*

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Stability of the OPH microneedle sensor performance

The ability to operate over prolonged periods with minimal deterioration in the current response represents another major requirement of minimally-invasive wearable biosensors.[23] As illustrated in Figure 4, the microneedle-based OPH biosensor demonstrated high reproducibility and stability. The precision test was performed using 50µM MPOx in artificial ISF by measuring 12 repetitive voltammograms at one-min intervals. As illustrated in Figure 4A, the OPH microneedle sensor displays a highly reproducible response, retaining its original current signal throughout this operation (RSD=2.5%, N=12). The stability of the OPH biosensor was evaluated by immersing the microneedle array in artificial ISF spiked with 50µM MPOx during over 2 h, recording the voltammograms at 10 min intervals (N=12). As illustrated in Figure 4B, the microneedle biosensor displays highly reproducible voltammograms over the entire period. A relative standard deviation of 3.74% is obtained for the oxidation peak current over this prolonged operation (n=12). These high stability and precision data indicate considerable potential of using the OPH microneedle biosensor for continuous transdermal OP monitoring. Whenever needed, the surface of the microneedle OPH biosensor can be easily renewed. The hollow microneedle enzyme biosensor offers convenient surface regeneration involving repacking of the CP and renewal of the reagent

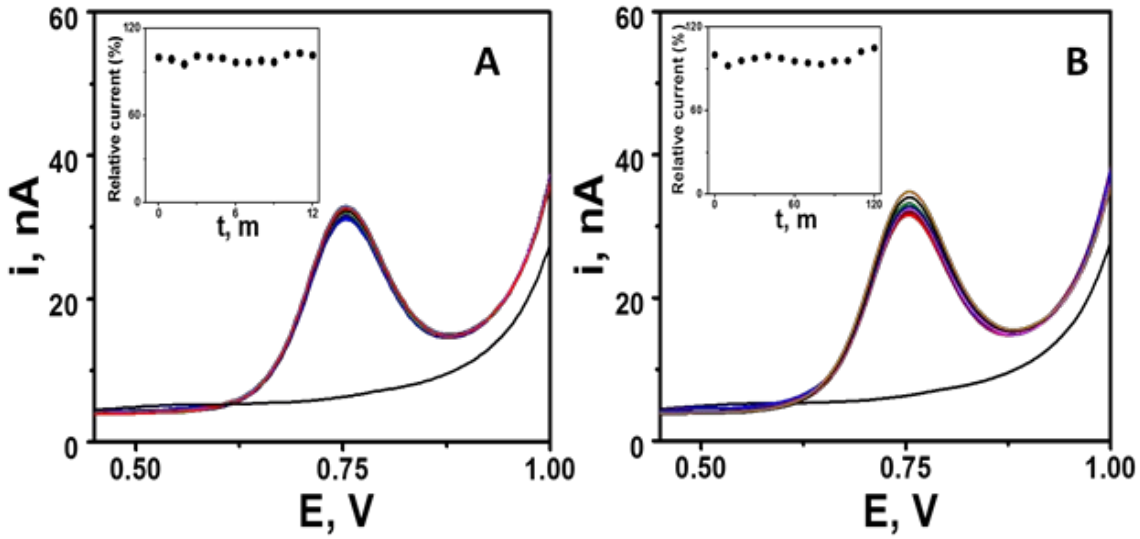

Figure 4: *Continuous monitoring of MPOx using OPH microneedle biosensor. A) Precision study of the MPOx response of the microneedle OPH sensor in artificial ISF; 12 repetitive measurements of 50µM MPOx performed in 1 min intervals, along with the background response. B) Stability study of the 50µM MPOx response of the microneedle OPH biosensor in artificial ISF; twelve repetitive measurements at 10 min intervals over a 2 h period.*

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layer. Four different calibration experiments for MPOx (20-180 µM), carried out using different renewed microneedle surfaces, resulted in highly repeatable voltammetric response and reproducible calibration plots (as in Figure 2A), with a 5% relative standard deviations of the individual slopes.

Ex-vivo mice skin test using OPH microneedle sensor

The applicability of developed OPH microneedle sensor was evaluated by employing it to mice skin exposed to MPOx. Based on the previously performed calibration curve, three different MPOx concentrations (50, 100 and 150 µM) were chosen to expose mice skin and assessed the performance of OPH microneedle sensor towards such skin analysis. The OP exposure on skin lasted for 20 min, followed by piercing the contaminated skin with the OPH microneedle sensor and recording the voltammograms within 30 sec.

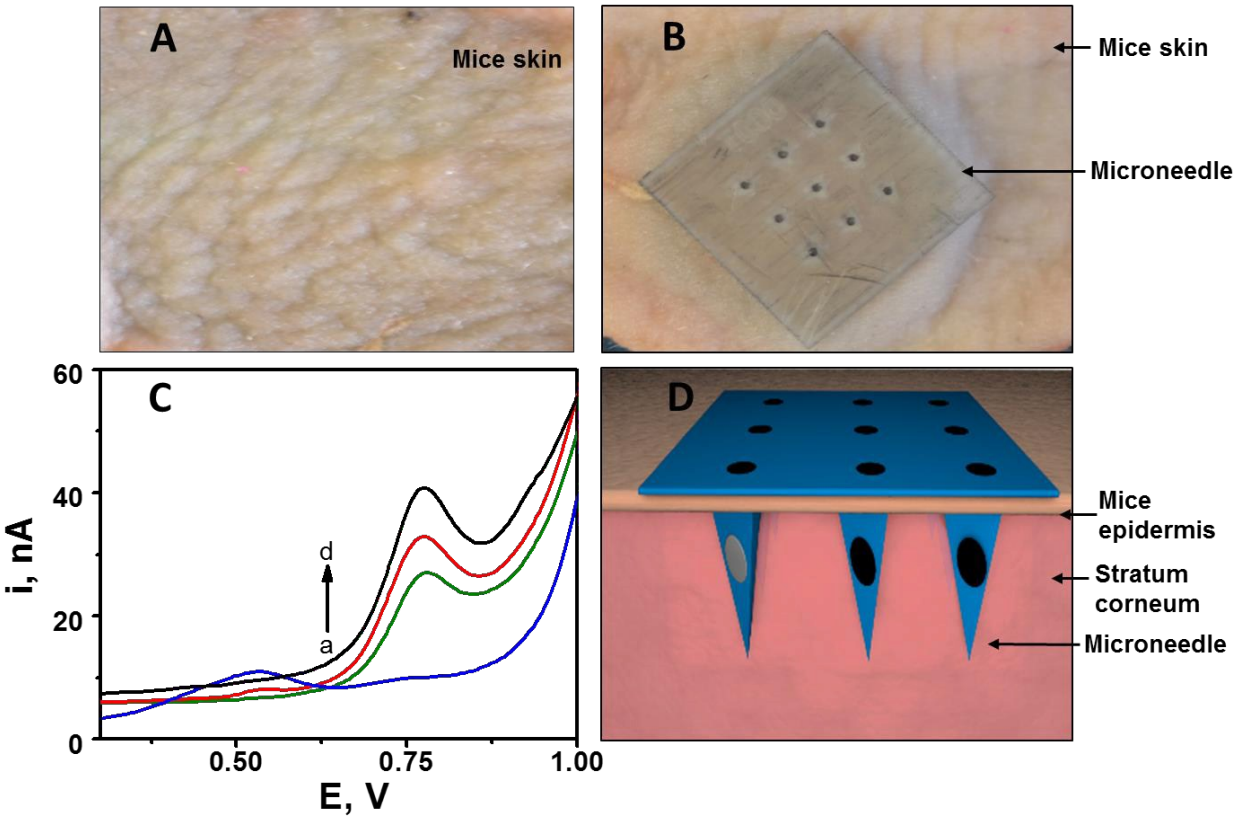

Figure 5: *Feasibility of transdermal detection of nerve agent in ex-vivo mice skin samples using the OPH microneedle sensor. A) Image of the mice skin before exposure to nerve agent; B) image*

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illustrating the OPH microneedle sensor piercing the mice skin to detect the nerve agent within the skin. C) Ex-vivo voltammetric detection of MPOx in contaminated mice skin samples using 0 (blue curve), 50 (green curve), 100 (red curve) and 150 (black curve) µM MPOx. D) Schematic of the microneedle sensor array piercing the skin. Conditions, as in Fig. 2A.

Figure 5 represents the transdermal detection of nerve agent using OPH microneedle sensor wherein images A and B correspond to the original mice skin before the OP exposure and to the needle penetrating the mice skin to detect the nerve agent, respectively. Figure 5C displays the SWV response of the microneedle biosensor in skin samples exposed to different MPOx concentrations (50, 100 and 150 µM) while Figure 5D shows schematic of the hollow microneedle piercing skin surface. It can be seen from Figure 5C that the OPH microneedle sensor can readily detect different MPOx levels within the skin, compared to the control (0 µM MPOx). These signals for the skin exposed to different MPOx concentrations (b-d) were compared to the MPOx calibration data (of Fig.2A). This comparison indicates good correlation with the 50 µM MPOx skin exposure signal resulted in 84% recovery whereas the 100 and 150 µM MPOx exposures led to 93 and 85% recoveries, respectively. The slightly lower values compared to solution-phase measurements of Figure 2a reflect the more viscous skin environment. The piercing microneedle sustained the skin penetration with no apparent damage. These mice skin MPOx detection data indicates the potential of the new microneedle biosensor approach for on-body measurements.

Conclusions

We demonstrated the first example of a microneedle-based OPH sensor for minimally-invasive transdermal monitoring of OP threats compounds. Such electrochemical monitoring of nerve agents has been accomplished by using minimally-invasive microneedle array (3×3) containing the CP transducers coated with OPH-Nafion reagent layer. While OPH biosensors commonly rely on amperometric detection, the new microneedle electrodes are based on information-rich voltammetric transduction of the response. Such voltammetric transduction is particularly attractive for enhancing the selectivity of minimally-invasive microneedle sensors. The resulting biosensor displays highly linear response for the MPOx model agent over the 20-180 µM range, along with effective discrimination against common electrochemical interferents and high stability and speed during prolonged in ISF solutions. *Ex-vivo* experiments using skin exposed to MPOx have proved the potential of the new biosensor for the minimally-invasive detection of chemical

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agents penetrating the skin. This represents the first example of a wearable (minimally-invasive) biosensor for detecting chemical threats or hazards. Such epidermal OPH sensor is minimally invasive, is easy-to-operate, and causes no burden to the wearer. Future efforts will involve further integration of the electronic interface and wireless transmission of the voltammetric output to a mobile device and detailed animal study. Use of microneedles of different lengths should offer better knowledge of the epidermal penetration of OP agents. Bendable microneedle array coupling rigid sharp tips with a soft base should be useful for tolerating deformations associated with the skin stretching during future applications.[20] The new microneedle detection concept can readily be extended toward transdermal monitoring of other chemical threats or hazards in connection to a variety of defense or occupational-health monitoring. The promising analytical results of this proof-of-concept study thus open the door for the development of a fast monitoring-decontamination close-loop system and of effective countermeasures for protecting our soldiers, farmers and civilians.

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