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TITLE: Real-Time In Vivo Measurement of Reactive Oxygen Species: Potential Measure to Mitigate Injury Sequelae of Hemorrhaging Warriors

PRINCIPAL INVESTIGATOR: Wayne Muraoka, PhD

CONTRACTING ORGANIZATION: Metis Foundation, San Antonio, TX

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Fort Detrick, Maryland 21702-5012

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Wayne Muraoka, PhD

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background & Rationale: Future conflicts that occur in anti-access and area denial (A2/AD) environments present unique challenges to prompt and expeditious medical evacuation for casualties. It is anticipated that combat casualty care will be necessary for prolonged periods, likely beyond the "golden hour". Hemorrhage, particularly from the torso and junctional regions, are deemed injuries to be potentially survivable. Resuscitative endovascular occlusion of the aorta (REBOA) and tourniquets provide temporary cessation of blood loss and are promising adjuncts in delaying acute mortality due to exsanguination. In austere environments, these hemorrhage control interventions allow for sustained perfusion of vital organs in the event of delayed evacuation. Nevertheless, 60 min is the established maximum for complete thoracic REBOA due to the risk of organ failure from ischemia- reperfusion injury (IRI). The hallmark of IRI is the robust production of reactive oxygen species (ROS) which occurs very early and causes tissue damage. If not immediately addressed, organ failure can ensue which is difficult to correct and has high mortality rates. Rapid and real-time measurement of ROS to assess the progression of IRI is extremely difficult because ROS is highly reactive and short-lived in the patient.

Hypothesis: In this proposal, we hypothesize that the continuous monitoring of ROS evolution by novel tools such as electrochemical biosensors within live tissues will provide real-time indication of IRI.

Aims & Study Design: In aim 1, the surface chemistry of the bioreagent will be optimized to enhance the biosensor's robustness and to improve its sensitivity and signal-to-noise ratio. The probe will also be miniaturized for field applications. The modified biosensor will be validated using freshly prepared ex vivo rat kidneys subjected to hypoxia-reoxygenation. In aim 2, the optimized electrochemical biosensor will be validated in a rat model of trauma, hemorrhage, and ischemia-reperfusion by implanting the biosensors into various tissues. The objectives of this aim are to determine whether ROS measurements correlate with blood chemistry markers of IRI and to determine whether ROS is a prognostic indicator of IRI for real-time decision support.

Innovation and Impact: Oxidative stress and excessive ROS accumulation are at the genesis of many pathological conditions. Because ROS production occurs very early in the progression of IRI, real-time detection and measurement of ROS can continuously monitor the casualty's condition and therefore facilitate casualty triage and treatment. However, current tools to evaluate ROS as an indicator of injury progression have poor spatial and temporal resolution due to the high reactivity and short half-life of ROS. Our planned approach of applying an electrochemical biosensor to ROS detection is innovative as it overcomes both temporal and spatial limitations providing continuous, real-time feedback from the tissue of interest. This proposal directly impacts the 2019 AIMM focus area by applying electrochemical biosensors for decision support in a deployed or operational environment to diagnose a military-relevant injury. Specifically, we address ROS detection to monitor IRI as a sequelae to non-compressible torso hemorrhage; however, the biosensor is broadly applicable to other research areas. For example, junctional tourniquets that are FDA approved inevitably lead to IRI. Additionally, compartment syndrome caused by extremity trauma can lead to amputation or sometimes death if a fasciotomy is not performed, and we anticipate that continuous ROS monitoring to assess the progression of IRI will greatly benefit the care of these casualties. Additionally, excessive ROS accumulation is associated with pathologic conditions resulting from cardiovascular disease, systemic inflammatory response syndrome, and ischemic stroke. Therefore, this electrochemical detection of ROS cross-cuts several DoD and civilian research areas and is not limited to hemorrhage control and IRI.

Positive results in Aim 1 and Aim 2 will provide a tractable tool to continuously monitored ROS evolution, and sequelae mitigation strategies (e.g. pharmacologic agents and REBOA modifications) can be further investigated in swine models with longer critical care phase. Ultimately, the development of novel tools to monitor ROS in live tissues, followed by the identification of strategies to ameliorate IRI, provide an opportunity for significant advancement in a field where interventions to reduce early mortality directly contribute to sequelae that result in late morbidity and mortality.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	21	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			

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1. INTRODUCTION

Oxidative stress and excessive ROS accumulation are at the genesis of many pathological conditions. Because ROS production occurs very early in the progression of IRI, real-time detection and measurement of ROS can continuously monitor the casualty's condition and therefore facilitate casualty triage and treatment. However, current tools to evaluate ROS as an indicator of injury progression have poor spatial and temporal resolution due to the high reactivity and short half-life of ROS. Our planned approach of applying an electrochemical biosensor to ROS detection is innovative as it overcomes both temporal and spatial limitations providing continuous, real-time feedback from the tissue of interest.

2. KEYWORDS

Ischemia-reperfusion injury, rat, animal model, reactive oxygen species, ROS, electrochemical biosensor, real-time, superoxide, hemorrhage, oxidative stress

3. ACCOMPLISHMENTS

What were the major goals of the project? (Goals to be accomplished and status.)

United States Army Institute of Surgical Research/Clarkson University Updates:

Specific Aim 1:

- STATUS: started Y1Q1, ongoing
- Aim 1: The surface of the bioreagent will be optimized to enhance the biosensor's robustness and to improve its sensitivity and signal-to-noise ratio. The probe will also be miniaturized for field applications. The modified biosensor will be validated using freshly prepared rat kidneys subjected to hypoxia-reoxygenation.

United States Army Institute of Surgical Research Updates:

Specific Aim 2:

- STATUS: started Y1Q3, ongoing. (Keep aims/tasks as formatted. Remove and write over these instructions and examples of all text in blue before submission. For each aim/task, update the status for the reporting period.)

What was accomplished under these goals? (Detailed progress and results.)

United States Army Institute of Surgical Research Updates:

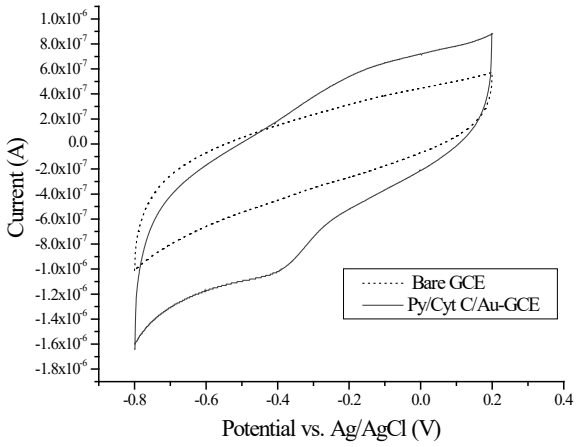
Specific Aim 1:

Animal protocols have been approved by local IACUC and ACURO.

United States Army Institute of Surgical Research/Clarkson University Updates:

Work during this time was performed to replicate the sensor design with consistent sensitivity for a broad range of superoxide concentrations (0.2 -1.4 μM) to facilitate translation of the technology. The superoxide biosensor fabricated on Au wire electrode using cytochrome C (cyt C) was develop with good reproducibility. While the use of self-assembled monolayers to attach the redox protein is a viable solution, this step is sensitive to a number of external factors such as humidity in the environment that can affect batch-to-batch reproducibility. We therefore investigate the use of alternative electrode coatings that could improve the surface chemistry, and that could also potentially be printed. This reporting period we have investigated the use of pyrrole (Py) is used as a surface immobilization matrix for the attachment of cyt c. Polypyrrole (PPy) is a conductive electroactive polymer that can be used as a matrix to entrap biomolecules providing increased bioactivity and sensitivity of biosensors. We evaluate the use of chemically synthesized gold (Au)-PPy on the electrode surface and immobilize cyt c within the polymeric nanostructure. To evaluate feasibility we first studied the electrochemical behavior of cyt c onto a glassy carbon electrode. The cyclic voltammetry shows well-defined peaks of cyt c onto the electrode confirming effective immobilization. Field-emission scanning electron microscopy shows uniform distribution of gold nanoparticles within a porous PPy matrix.

A



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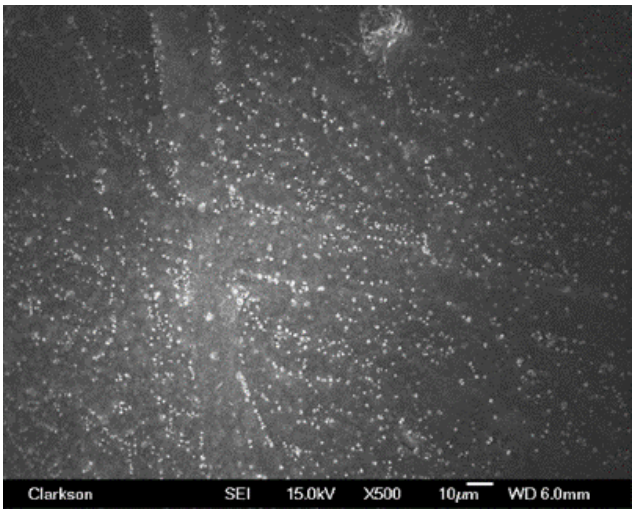
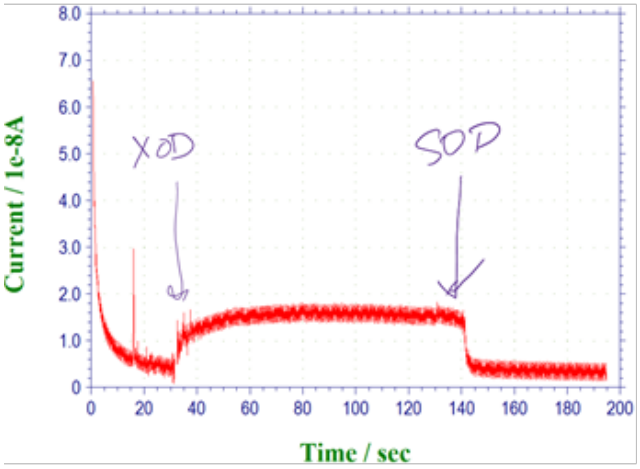


Figure 1. Characterization of biosensor with PPy to immobilize the biorecognition element. (A) Cyclic voltammograms of bare glassy carbon electrode (GCE) and GCE-Au-PPy-Cyt c electrode in 0.1M phosphate buffer at pH 7.4 at a scan rate of 50 mV Sec^{-1}. (B) Field-emission scanning electron microscopy (FE-SEM) of the Au-PPy-GCE electrode.

The technology was successfully transferred to Dr. Muraoka's laboratory where the biosensor was fabricated and evaluated with ex vivo samples. In initial experiments, we focused on simple, proof-of-concept experiments to demonstrate practical application and validation of the biosensors. Platelet activation is a critical step to initiate hemostasis in actively bleeding patients and platelet transfusion to these patients is life-saving. Methods to test platelet activation and function is an active area of research because stored platelets have diminished activity. Using the biosensor, we monitored superoxide anion ($O_2^{\cdot-}$) evolution in washed platelets after activation with thrombin. We show increased signal after addition of thrombin in fresh platelets, but significantly reduced signal in platelets stored for seven days. This application of the biosensor has the potential to monitor platelet function during storage and identify platelet units with high functional capacity.



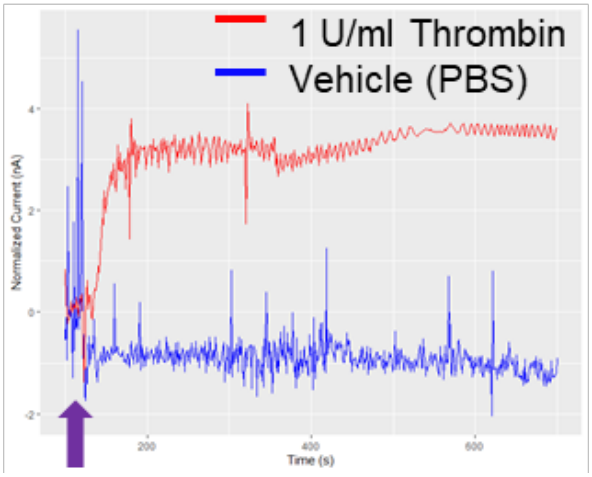


Figure 2. Successful technology transfer to the ISR showing fabrication and application of the sensor. (A) Specific detection of $O_2^{\cdot-}$. Arrows show time-point where $O_2^{\cdot-}$ was chemically synthesized by introducing Xanthine Oxidase (XOD) and then consumed by adding Superoxide Dismutase (SOD). (B) Application of the biosensor to evaluate platelet function. Our initial attempts to apply the biosensor to combat casualty care was to monitor $O_2^{\cdot-}$ evolution from washed platelets stimulated with thrombin. Purple arrow indicates introduction of thrombin (red trace) or vehicle (blue trace) to the electrochemical cell.

Key Findings or Accomplishments:

- Immobilization of the bio-recognition element with PPY is feasible and allows uniform distribution of Au nanoparticles within the PPY matrix.
- Platelet activation induces $O_2^{\cdot-}$ release which can be monitored with the biosensor.

Specific Aim 2:

Follow the same instructions as above.

What opportunities for training and professional development has the project provided?

Clarkson University Updates: Training: Aaditya Deshpande (Graduate Student) continued his training under the mentorship of Dr. Silvana Andreescu. This training was critical in the optimization and fabrication of the biosensor using PPy.

United States Army Institute of Surgical Research Updates:

Professional development: Cheresa Calhoun (Research Associate) continues to enhance her skill sets to now include rodent surgical procedures.

How were the results disseminated to communities of interest?

United States Army Institute of Surgical Research/Clarkson University Updates: We have published a peer-reviewed article entitled “Electrochemical sensors for oxidative stress monitoring” in the journal Current Opinions in Electrochemistry. https://doi.org/10.1016/j.coelec.2021.100809.

Plans for the next reporting period to accomplish the goals

United States Army Institute of Surgical Research Updates: We are currently optimizing the method to prepare the kidneys to validate the sensor during hypoxia-reoxygenation. Initial experiments produced equivocal results likely due to cell viability once the kidney was excised. We plan to utilize the alternative strategy that was presented in the proposal.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Clarkson University Updates: Uniform immobilization of biorecognition elements with PPy has applications beyond monitoring reactive oxygen species. For example, monoclonal antibodies can be immobilized for the detection of specific pathogens or compounds. Thus, the techniques used in this project can be generally applied to other sensor designs.

What was the impact on other disciplines?

United States Army Institute of Surgical Research Updates: Our initial proof-of-concept experiments with platelets demonstrated a practical application of this technology that can be used by blood banks. Rapid evaluation of platelet function may benefit transfusion outcomes in actively bleeding patients.

What was the impact on technology transfer?

United States Army Institute of Surgical Research Updates: The initial technology for biosensor fabrication has been transferred to the ISR (government) to evaluate its application in combat casualty care. The application of these sensors to monitor ischemia-reperfusion is novel and is being reviewed for an invention disclosure, pending experiment outcomes in Specific Aim 2.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS

IMPORTANT REMINDER – Award recipient organization is required to obtain prior written approval from the awarding agency Contracting/Grants Officer whenever there are significant changes in the project or its direction such as significant change in scope or the Statement of Work (e.g. removal, change, or addition of aims/tasks or animal model change), change in PI or key personnel, reduction of 25% FTE, or significant change in budget.

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

United States Army Institute of Surgical Research/Clarkson University Updates: COVID-19 travel restrictions have limited collaboration interactions and training of Dr. Muraoka's team in the fabrication and operation of the biosensors. Nevertheless, Dr. Muraoka's laboratory is now able to fabricate the biosensors and have performed initial experiments with them.

United States Army Institute of Surgical Research Updates COVID-19 has created a back log for animal studies in the vivarium. Given that we are in the early stages of technology transfer, this is not an issue until animal studies commence. We will provide the vivarium with enough time to schedule our experiments.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

United States Army Institute of Surgical Research Updates:

TOTAL PROTOCOL(S): 1

PROTOCOL (X of Y total):
IACUC Protocol Number: A-20-028
ACURO Protocol Number: DM190422.e001
Protocol
Protocol Site: USAISR

Protocol Title: Real-Time Measurement of Reactive Oxygen Species During Hemorrhage and Ischemia-Reperfusion Injuries in Rats (*Rattus norvegicus*)
Number of Animals Approved for Use: 23
IACUC INITIAL APPROVAL DATE: 7/6/2020 (expires 7/6/2023)
ACURO INITIAL APPROVAL DATE: 8/21/2020
RENEWAL APPROVAL DATES:

- None.

AMENDMENTS:
- None.

ADVERSE EVENTS OR UNANTICIPATED PROBLEMS:
- None.

Significant changes in use of biohazards and/or select agents

Not applicable.

6. PRODUCTS

Journal publications

United States Army Institute of Surgical Research/Clarkson University Updates:

1. Deshpande A, Muraoka W, Andreescu S. Electrochemical sensors for oxidative stress monitoring. Curr Op Electrochem. 2021; 29:100809. https://doi.org/10.1016/j.coelec.2021.100809.
    a. Review
    b. Published
    c. Directly related to SOW, specific aim 1
    d. DoD funding acknowledged

Books or other non-periodical, one-time publications

Nothing to Report.

Other publications, conference papers, and presentations

Nothing to Report.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

United States Army Institute of Surgical Research Updates:

Name: Wayne Muraoka
Project Role: PI
Researcher Identifier: 0000-0002-7960-7176
Nearest person month worked: 4
Contribution to Project: Wrote and obtained approvals for animal protocol. Transitioned technology to ISR. Established working biosensors at ISR. Performing studies for ex vivo ROS monitoring.

Clarkson University Updates:

Name: Silvana Andreescu
Project Role: Co-PI
Researcher Identifier: 0000-0003-3382-7939
Nearest person month worked: 2
Contribution to Project: Overseeing electrode modification.

Name: Aaditya Deshpande
Project Role: Graduate Student
Researcher Identifier:
Nearest person month worked: 9
Contribution to Project: Performing experiments for electrode modification.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHART

9. APPENDICES

Real-Time In Vivo Measurement of Reactive Oxygen Species: Potential Measure to Mitigate Injury Sequelae of Hemorrhaging Warriors

Proposal Log #: DM190422, Award #: W81XWH2020054

PI: Wayne Muraoka, PhD **Org:** The Metis Foundation **Award Amount:** $324,972

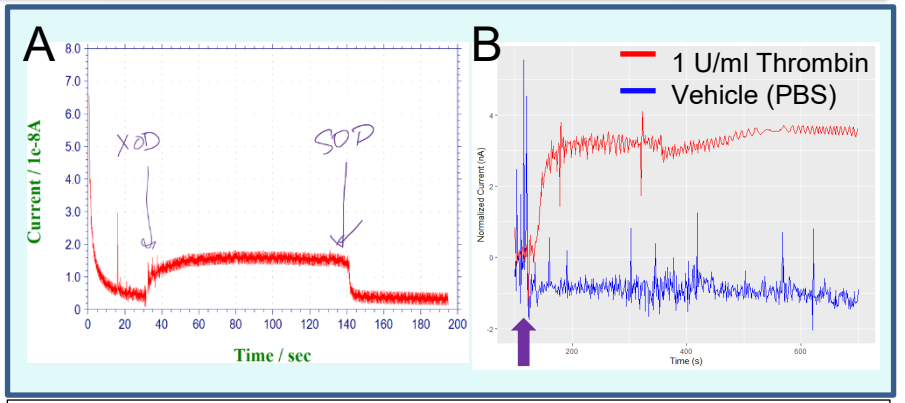
Study/Product Aim(s)

•Aim 1: The surface of the bioreagent will be optimized to enhance the biosensor's robustness and to improve its sensitivity and signal-to-noise ratio. The probe will also be miniaturized for field applications. The modified biosensor will be validated using freshly prepared rat kidneys subjected to hypoxia-reoxygenation.

• Aim 2, The optimized biosensor will be validated in a rat model of ischemia-reperfusion injury (IRI). The objectives of this aim are to determine whether reactive oxygen species (ROS) measurements correlate with biomarkers of IRI and to determine whether ROS is a prognostic indicator of IRI for real-time decision support.

Approach

Oxidative stress and excessive ROS accumulation are at the genesis of many pathological conditions. Because ROS production occurs very early in the progression of IRI, real-time monitoring of ROS can continuously provide information of the casualty's condition and therefore facilitate casualty triage and treatment. However, current tools to evaluate ROS as an indicator of injury progression have poor spatial and temporal resolution due to the high reactivity and short half-life of ROS. Our planned approach of applying an electrochemical biosensor to ROS detection is innovative as it overcomes both temporal and spatial limitations providing continuous, real-time feedback from the tissue of interest.



Accomplishment: Successful technology transfer to the ISR showing fabrication and application of the sensor. (A) Specific detection of superoxide anion ($O_2^{\bullet-}$). Arrows show time-point where $O_2^{\bullet-}$ was chemically synthesized by introducing Xanthine Oxidase (XOD) and then consumed by adding Superoxide Dismutase (SOD). (B) Application of the biosensor for acute traumatic coagulopathy. Our initial attempts to apply the biosensor to combat casualty care was monitor $O_2^{\bullet-}$ evolution from washed platelets stimulated with thrombin. Purple arrow indicates introduction of thrombin (red trace) or vehicle (blue trace) to the electrochemical cell.

Timeline and Cost

Activities CY	20	21
Local IACUC and ACURO approval (**USAISR**)		
Optimize sensor characteristics (**Clarkson University**)		
Ex vivo validation (**USAISR**)		
In vivo validation (**USAISR**)		
Estimated Budget ($K)	**$211K**	**$114K**

Updated: October 20, 2021

Goals/Milestones

United States Army Institute of Surgical Research Updates:

CY20 Goal – Project initiation and approvals

☑ Obtain Local IACUC and ACURO approvals

Clarkson University Updates:

CY21 Goals – Sensor optimization and training

☑ Improve surface chemistry and evaluate electrode printing methods

☑ Training of Muraoka lab for sensor operation

☐ Ex vivo evaluation of the sensor – in progress

United States Army Institute of Surgical Research Updates:

CY22 Goal – Sensor validation

☐ Validate sensor in a rat model of IRI and data analysis

☐ Presentation and publication of optimized sensor

Comments/Challenges/Issues/Concerns

- COVID-19 has created a back log in our vivarium. Given that we are still in the early translational stage, this is not a big concern.

Budget Expenditure to Date: September 30, 2020 – July 1st, 2021

Projected Expenditure for Years 01-02: $324,972

Actual Expenditure: $64,514.88

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Electrochemistry

Review Article

Electrochemical sensors for oxidative stress monitoring

Aaditya S. Deshpande[1], Wayne Muraoka[2] and Silvana Andreescu[1]

Abstract

Electrochemical sensors are ideally suited for the detection of reactive oxygen and nitrogen species (ROS and RNS) generated during biological processes. This review discusses the latest work in the development of electrochemical microsensors for ROS/RNS and their applications for monitoring oxidative stress in biological systems. The performance of recent designs of microelectrodes and electrode materials is discussed along with their functionality in preclinical models of drug efficacy, mitochondrial distress, and endothelial dysfunction. Challenges and opportunities in translating this methodology to study the pathophysiology associated with various diseases are discussed.

Addresses

[1] Department of Chemistry and Biomolecular Science, Clarkson University, Potsdam, NY 13699, USA

[2] U.S. Army Institute of Surgical Research, Blood and Shock Resuscitation, Fort Sam Houston, TX 78234, USA

Corresponding author: Andreescu, Silvana (eandrees@clarkson.edu)

Current Opinion in Electrochemistry 2021, **29**:100809

This review comes from a themed issue on **Bioelectrochemistry**

Edited by **Pankaj Vadgama**

For a complete overview see the Issue and the Editorial

Available online 14 July 2021

https://doi.org/10.1016/j.coelec.2021.100809

Keywords

Oxidative stress, ROS/RNS, Nitric oxide, Hydrogen peroxide, Superoxide.

Introduction

Reactive oxygen and nitrogen species (ROS and RNS) are formed by redox reactions of molecules that contain oxygen or nitrogen. Reactive oxygen species include superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid/hypochlorite ($HOCl/^{-}OCl$), and singlet oxygen ($^1O_2$) [1,2]. Reactive nitrogen species include nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$), and peroxynitrite ($ONOO^-$). Reactive oxygen/nitrogen species are continuously produced during normal cell processes like oxidative phosphorylation, catabolism of fatty acids, phagocytosis, breakdown of macromolecular compounds, and protein folding. Classified as radical (e.g. $O_2^{\bullet-}$, $NO^{\bullet}$, and $OH^{\bullet}$) or non-radical (e.g. HOCl and H_2O_2), each type of ROS has a different reactivity rate, biological activity, and role within the cell [3,4]. Collectively, both radical and nonradical ROS contribute to the overall oxidative burden of the cell [2,4], and, when in excess, the highly reactive nature of ROS/RNS can damage cell components, leading to oxidative stress [5]. While the significance of ROS is in general well recognized, some aspects of ROS in distinguishing the physiological and pathological processes are still debated.

Despite the importance of ROS/RNS, there is still a lack of suitable analytical tools to selectively monitor ROS/RNS evolution to address the cell's oxidative status in situ. Using custom designed microelectrodes, electrochemistry provides unique opportunities to detect reactive species in living tissues [6], thereby providing direct and real-time evidence of ROS levels with high spatial resolution [7–9]. In this review, we discuss electrochemical detection methods and microsensors for ROS/RNS, with focus on H_2O_2, NO and $ONOO^-$ and $O_2^{\bullet-}$. Finally, we conclude with a perspective on future advances of this technology for the development of electrochemical probes for real-time monitoring of these species in biological systems as well as translational aspects for medical applications.

Cell oxidative stress: relevance and significance

Reactive oxygen species play an essential role in redox signalling, allowing the cell to rapidly adapt to environmental or nutritional perturbations [3,10,11]. Under homeostatic conditions, both enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (e.g., glutathione, thioredoxin, ascorbate) antioxidant mechanisms tightly regulate ROS levels and prevent excess accumulation [11,12]. Dysregulation of the balance between pro-oxidants and antioxidants is associated with physiological and developmental derangements. Insufficient H_2O_2 restricts neuron growth, induces stem cell quiescence, and thwarts wound healing, while insufficient $O_2^{\bullet-}$ impairs

immune cell clearance of pathogens [13–15]. However, excessive ROS damages DNA, proteins, and lipids, and can lead to cell death, tissue damage, and if not corrected, organ failure [16–18]. Indeed, oxidative damage to lipid (8-isoprostaglandin F2α and malondialdehyde), DNA (8-hydroxy-deoxyguanosine), and protein (3-nitrotyrosine) are often used as biomarkers of oxidative stress in clinical samples [19,20]. Oxidative stress is an imbalance of cellular redox where a pro-oxidant state is favoured and is implicated in a myriad of diseases [21,22]. Different types of ROS can impact cell physiology and oxidative stress in different ways, and it is important to measure individual species to understand the impact of a specific ROS within each (patho)physiologic setting [2]. Therefore, monitoring a specific ROS in different disease conditions and experimental treatments is necessary to investigate disease mechanisms and drug efficacy. However, ROS are highly reactive and extremely short lived in the body, making its direct measurement in live tissues and organisms difficult.

Biomarkers have the limitation that their accumulation or removal will alter the quantity of the measured analyte but may not correspond to nascent ROS evolution. Furthermore, biomarker measurements do not provide insight into the type of ROS that is dysregulated, and it is difficult to deconvolute the consequence from the cause of oxidative stress. Methods to measure specific types of ROS in tissues were described for electron paramagnetic resonance and ultraweak photon-emission spectroscopy [23,24]; however, their application for *in vivo* ROS monitoring are hampered by high cost and low temporal resolution. Therefore, there is a need to develop tools that have an extremely rapid response time, are sensitive and selective to individual species, and capable of real time detection. The accurate measurement of these species is still a bottleneck in understanding their physiological functions and a universal technique that can detect the wide variety of radicals is not available [25].

Electrochemical sensors and biosensors for ROS/RNS detection

Monitoring ROS/RNS using electrochemistry provides a valuable approach to quantifying oxidative stress generated by these species in situ and can help elucidate their biological roles. Although a variety of electrochemical sensors have been reported, relatively few studies demonstrate their use in cells or biological systems. The use of glassy carbon electrodes is common in literature; however, its bulky size restricts use to proof-of-concept work to develop new chemistries, and it is not suitable for live tissues. Smaller size electrodes that can measure ROS in the proximity of cells are most suited to explore biological mechanisms in situ. Therefore, this review focusses primarily on microelectrodes that have been used to measure reactive species at the cellular or tissue level. These include carbon fibre microelectrodes (CFME) with sizes from 5 to 10 μm and gold or platinum wire microelectrodes with a diameter of ~100 μm.

Reactive oxygen/nitrogen species can be measured using microelectrodes functionalized with chemical or biological coatings. Chemical sensors provide a direct measure of the reactive species at their characteristic potentials. Common examples are those measuring the oxidation of NO at ~0.8 V vs Ag/AgCl, or the oxygen/superoxide redox couple at ~ -0.33 V versus saturated hydrogen electrode (NHE) [9]. Pioneering work done by Amatore and Arbault [9], Amatore et al. [26], and Hu et al. [27, 28] demonstrated the use of platinized carbon microelectrodes (~10 μm diameter) for monitoring ROS/RNS species produced by single cells [9,26–28] and their ability to measure reactive species inside single phagolysosomes of living macrophages using a four step chronoamperometric method [28]. Recent advances involve modification of microelectrodes with catalytic materials to enhance the detection sensitivity and tailor selectivity. In contrast, biological sensors are protein-functionalized electrodes that contain a redox protein immobilized at the electrode surface to selectively recognize the targeted species and convert the biorecognition into an electrochemical redox signal. A common example is the use of cytochrome *c* (Cyt C) as molecular recognition and electron transfer mediator for $O_2^{\cdot-}$ measurements [29]. In these sensors, the immobilized Cyt C reacts with $O_2^{\cdot-}$; the protein is then oxidized by direct electron transfer to/from the electrode, generating a biocatalytic current that is proportional to the $O_2^{\cdot-}$ concentration. Because biological sensors take advantage of the selectivity of biomolecules, they tend to be more selective. However, they require immobilization of the biomolecule onto the microelectrode surface, and the long-term stability of these sensors might be an issue. Table 1 provides an overview of microelectrode platforms for measuring superoxide $O_2^{\cdot-}$, H_2O_2. The following sections discuss the most recent representative examples of microelectrochemical sensors for measurements of ROS/RNS in biological systems.

Electrochemical sensors for H_2O_2 and superoxide radicals

The electroactive H_2O_2 can be detected electrochemically using a chemically modified electrode [6]. Xu and co-workers [33] modified a CFME with Au nanocones and a synthetic molecular receptor having affinity towards H_2O_2. The small size of the CFME coupled with the selectivity of the synthetic receptor enabled measurements of H_2O_2 in a single drop of blood. Measurements were performed by differential pulse voltammetry (DPV) in the range −0.5–0.6 V *vs* Ag/AgCl electrode and the sensor was able to measure H_2O_2 in

Table 1

Details of some electrochemical sensors and biosensors and electrode modifications for detection of ROS/RNS released from cells and tissues.

#	ROS/RNS	Electrochemical technique	Electrode materials	Working electrode	LOD	Biological system	Reference
1	H_2O_2	Fast scan cyclic voltammetry	1,3-phenylenediamine	CFME	20 μM*	Rat brain	[30]
2	H_2O_2	Chronoamperometry	Pt–Pd bimetallic nanocoral	CFME	0.42 μM	A549 living cells, milk	[31]
3	H_2O_2	Amperometry, CV	Hemoglobin, SWCNT	CFME	0.23 μM	HePG2 cancer cells	[32]
4	H_2O_2	Amperometry	Au–Pd alloy NPs, graphene quantum dots	CFME	500 nM	Clinical breast cancer tissue	[33]
5	H_2O_2	Amperometry	Pt NPs, Nafion, PPD	CFME	0.53 μM	*In vitro*	[34]
6	H_2O_2	Amperometry	Platinized silica nanoporous membranes	CFME or ITO	0.01 mM	Rat brain	[35]
7	H_2O_2,	Chronoamperometry	Heat treatment to create nanopores to improve catalytic performance	Heat-treated CFME	1 μM	*In vitro*	[36]
8	H_2O_2	Chronoamperometry	Core-shell 2D VS_2,@VC@N-doped carbon sheets decorated by Pd NPs	CFME	50 nM	MCS-7 cancer cells, and breast cancer tissue	[37]
9	H_2O_2	Chronoamperometry	Pt–Pd NPs, graphene oxide	CFME	0.3 μM	Raw 264.7 cells secretion	[38]
10	H_2O_2	Chronoamperometry	Au–Ag bimetallic NPs/polydopamine	CFME	0.12 μM	HepG2 cells	[39]
11	HClO/ClO^-	DPV	Graphene oxide, carbon nanotubes, MBS	CFME	0.5 μM	Body fluids	[40]
12	O_2^{-}	Chronoamperometry	MWCNTs, Ionic Liquid-Br, SOD, Prussian Blue NPs	CFME	0.42 μM	Alzheimer rat brains	[41]
13	O_2^{-}	DPV with ratiometric signal output	Diphenylphosphonate-2-naphthol ester, methylene blue SWCNTs	CFME	2 μM	Rat brain	[42]
14	NO	DPV	NiTSPc/nafion	CFME	0.34 μM	Zebrafish intestine	[8]

a-NSGF, taurine-functionalized graphene foam; AgNPs, silver nanoparticles; APTES, (3-aminopropyl) triethoxysilane; AuNP, gold nanoparticles; BA, 5-(1,2-dithiolan-3-yl)-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)pent-anamide; BBY, Bismarck Brown Y; CFME, carbon fiber microelectrode; CNT, carbon nanotubes; CTS, chitosan; CV, cyclic voltammetry; Cyt C, cytochrome C; D-cell, plastic disposable carbon-based electrochemical cell; DNA, deoxyribose nucleic acid; FTO, fluorine-doped tin oxide; HTCMP, hollow tubular conjugated organic microporous polymer; ITO-PET, indium tin oxide supported on poly-ethylene-terephthalate foil; MPNS, microporous polymeric nanospheres; MWCNTs, multiwalled carbon nanotubes; PDDA, poly(diallyl dimethyl ammonium chloride); Poly(5A1N), electropolymerized 5-amino-1-naphthol; PSS, polystyrene sulfonic acid; rGO, reduced graphene oxide; XG, xerogel.

the 0.5−400 μM range [43]. de Meira et al. [44] used a disposable plastic carbon-based electrochemical cell with a chemically modified electrode coated with Ag nanoparticles and δ-FeOOH. The reduction of H_2O_2 catalyzed by Ag nanoparticles (NPs) lead to increased sensitivity for H_2O_2 detection in fetal bovine serum. Nonenzymatic detection of H_2O_2 is achieved with electrodes functionalized with chemical mediators such as Prussian blue (PB), used alone or in composite forms with Au nanoparticles or graphene oxide. A PB-AuNPs-graphene oxide deposited on a GCE enabled detection of H_2O_2 down to 1.3 μM [45]. Similar strategies can be used to increase selectivity of CFMEs (Table 1). Most sensors use Pt-based structures that take advantage of the catalytic activity of PtNPs for H_2O_2; these are often combined with Au, Ag, carbon nanotubes, or graphene oxide for enhanced performance. In some cases, the growth and self-assembly of a multidimensional structure on the surface of CFMEs reduces the oxidation potential minimizing interferences. A CFME functionalized with VS_2, @VC@N-doped carbon sheets decorated by PdNPs enabled detection of H_2O_2 at −0.05 V with no interferences from dopamine, uric acid, ascorbic acid, and nitrite [37].

The standard redox potential of the $O_2/O_2^{\cdot-}$ redox couple is between 330 mV and 140 mV versus NHE and thus this can be determined by direct electrochemical oxidation using platinized microelectrodes, or electrodes modified with nitrogen-doped carbon AgNPs [46] or porous carbon networks [47]. To improve selectivity, a common approach is to immobilize Cyt C or superoxide dismutase onto AuNP-functionalized microelectrodes [6]. A recent trend is to use enzyme mimetic materials such as $MnTiO_3$ microdiscs [48], manganese phosphate [49], or nanostructured Mxenes [50] and graphene/AgNP/CeO_2/TiO_2 [51] as alternative to natural enzymes. However, specificity of measurements is not always demonstrated, raising questions about the accuracy of such configurations. Thick-films Cyt c−based nanoporous gold electrodes with a detection limit of 1.9 nM and a sensitivity of 1.9 nM.nM^{-1}cm^{-2} enabled the online detection of $O_2^{\cdot-}$ in skeletal muscle tissue [52].

Electrochemical sensors for nitric oxide and peroxynitrite

Nitric oxide is a highly diffusible short-lived species, which can interact with $O_2^{\cdot-}$ to form peroxynitrite, a highly reactive and toxic species that can damage DNA, proteins, and lipids. Because NO has reduced stability, NO sensors must have a short response time, be sensitive, and have a wide linearity range. The electrooxidation of NO takes place at a potential >0.8V vs Ag/AgCl that overlaps with the oxidation potential of other electroactive species. To prevent interferences, CFMEs are commonly functionalized with blocking membranes such as Nafion [8], o-phenylene diamine (o-PD), and chitosan [8,53]. Electrochemical NO sensors have been reviewed by Brown and Schoenfisch [54] (see Figure 1).

Long-term electrochemical measurement of NO released from cultured pro-inflammatory macrophages was demonstrated using an Pt disk electrode (6 mm diameter) modified with an electropolymerized 5-amino-1-naphtol (Poly(5A1N)) and fluorinated xerogel to prevent degradation (Figure 2) [55]. The xerogel provided permselective properties imparting selectivity and preventing biofouling. A detection limit of 1 nM and a dynamic range 0.01−10 μM was reported. Detection of NO in human serum (detection limit of 52 nM and linear range of 0.25−40 μM) was reported with an electrode coated with reduced graphene oxide and PtNPs [56]. A microsensor enabling detection of $NO^{\cdot}$ in the presence of H_2O_2 in static or flow conditions was achieved with a dual-electrode set up. Poly(eugenol) coating enhanced the selectivity of the Pt electrode and was superior to bare Pt and Pt−Pt black [57].

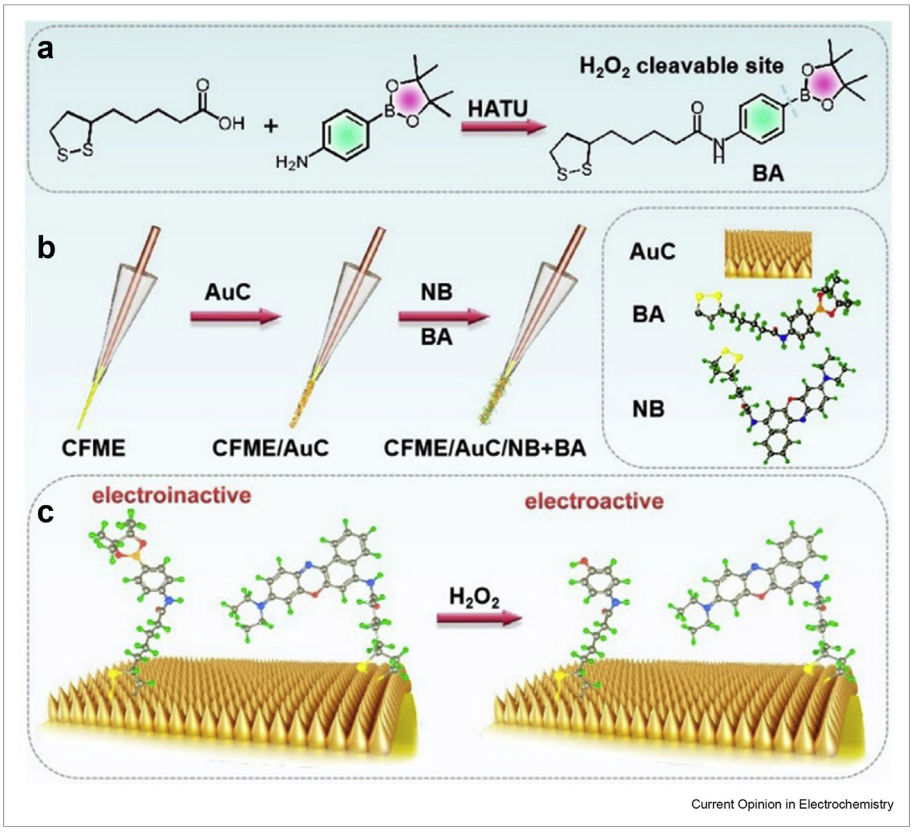
Recent developments in microelectrode design integrate microelectrodes and wireless monitoring. Using a flexible transient electrode, real-time monitoring of NO over 5 days was recorded in the hearth and joint cavity of rabbits [58]. The implantable sensor consisted of a biocompatible electrode constructed from polylactic acid and poly(trimethylene carbonate), an ultrathin Au membrane, and a poly(eugenol) film. This sensor had a detection limit of 0.97 nm and a 0.01−100 μM linear range (see Figure 3).

Peroxynitrite, the primary product formed in the fast reaction of superoxide radicals with NO, is an important but difficult to measure RNS [59]. The formal potential of $ONOO^-/ONOO^{\cdot}$ is 0.27 V vs SSCE [60]. Electrochemical detection of transient concentrations of peroxynitrite was achieved with platinized, or nanostructured microelectrodes modified with conjugated Mn complexes (e.g., tetraaminophthalocyanine manganese (II) [61], MnO_2-Hemin [62] and PEDOT-Hemin [63] layers, and microporous polymeric nanospheres [64]) acting as electrocatalytic sites. Recent efforts are dedicated to simultaneous detection of multiple ROS/RNS released by cells by custom-designed microfluidic devices [65] (see Figure 4) and ratiometric measurements [66]. Such measurements can be effective at determining multiple ROS/RNS species simultaneously and take into account issues of cross-reactivity. This approach is highly suited for the high throughput monitoring of cells.

Biological applications and translational aspects

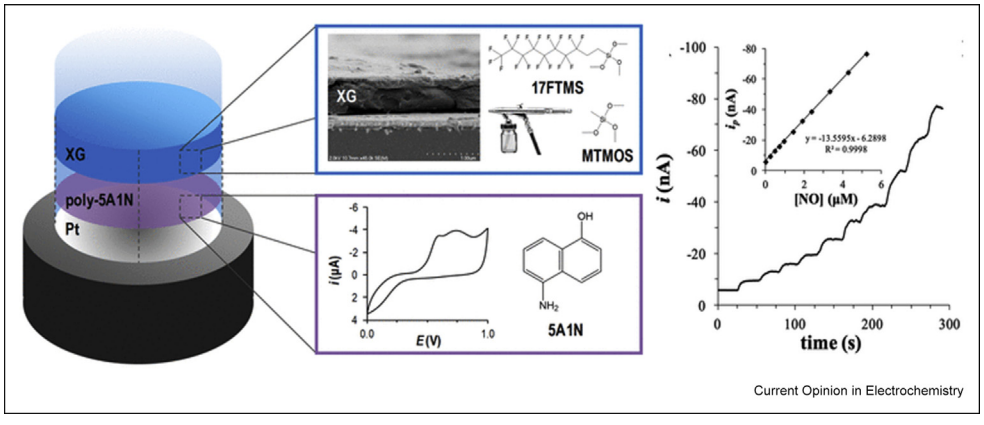
Accurate ROS measurements are important to understand the relationship between oxidative stress and

Figure 1



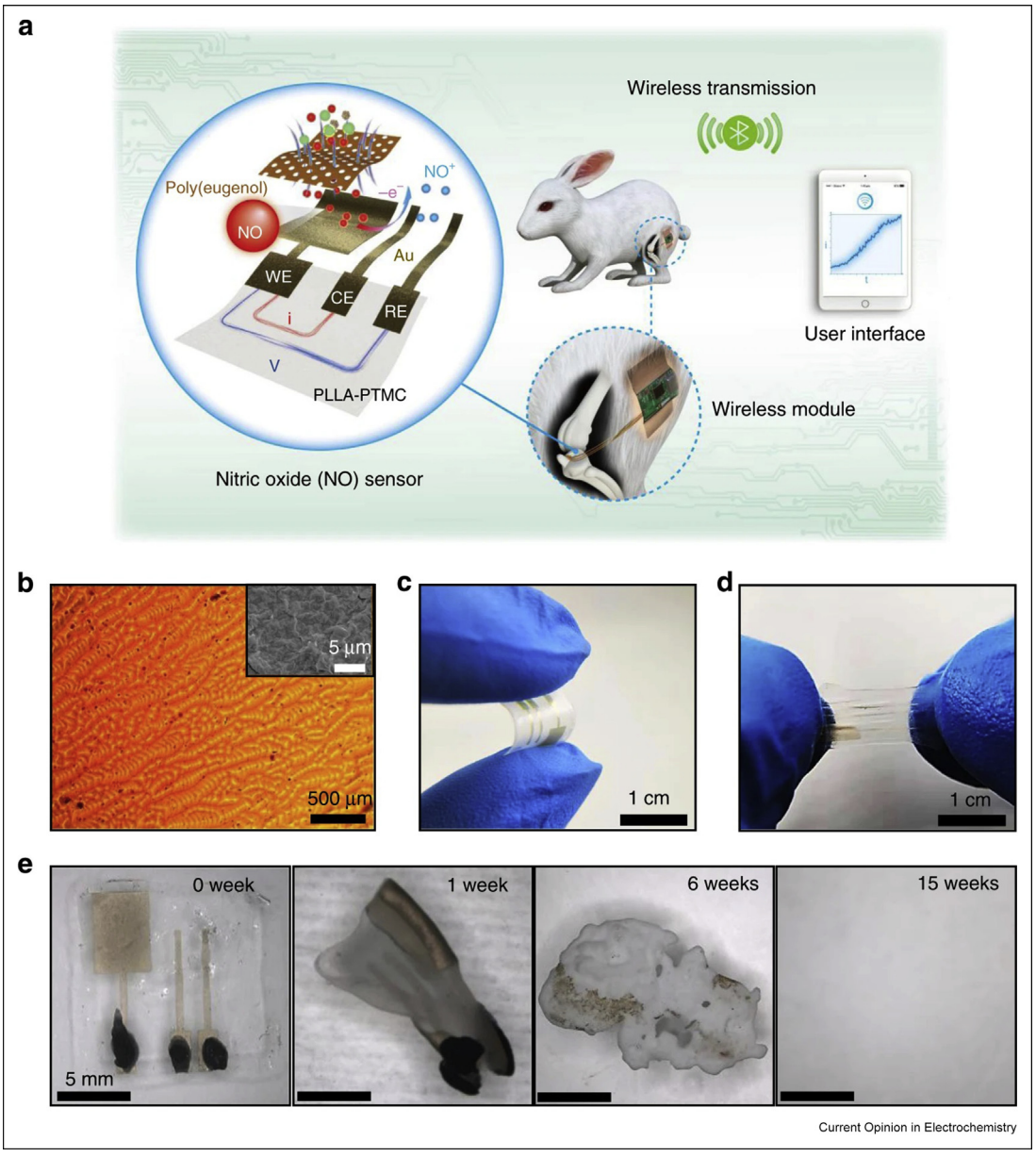
Example of electrochemical H_2O_2 sensors used using a chemically modified CFME. Reproduced with permissions (Dong et al. [43] with permission).

Figure 2



Example of NO sensor coated with gel to prevent degradation in biological medium (with permission from the study by Brown and Schoenfisch [55]).

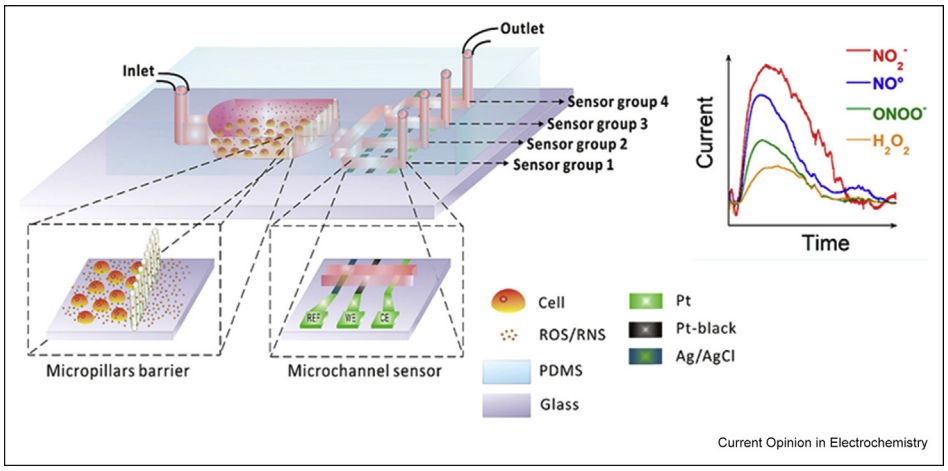
Figure 3



Flexible and transient NO sensor: **(a)** Illustration of sensor design composed of a bioresorbable copolymer of poly(L-lactic acid) and poly(trimethylene carbonate (PLLA–PTMC) substrate, Au nanomembrane electrodes, and a poly(eugenol) thin film. NO concentration was measured through amperometry. The sensor can continuously detect NO concentrations in vivo and transmit the data to a user interface through a customized wireless module. **(b)** Optical image of the surface morphology of Au electrodes and SEM image of the surface morphology. **(c)** NO sensor under bending. **(d)** NO sensor in a stretched state. **(e)** Images at various stages (0, 1, 6, and 15 weeks) of accelerated degradation of a transient NO sensor in phosphate-buffered saline (with permission from Ref. [58]).

disease. Oxidative stress underlies cardiovascular diseases by impairing endothelial cell function, thereby influencing vascular tone and inflammation [48,67]. How the mechanical forces from blood flow and smooth muscle contraction alter ROS production was addressed using a flexible electrochemical sensor [68]. This sensor allowed for the attachment of cells onto a compliant surface. By simulating *in vivo* conditions of mechanical stress, it was shown that circumferential stretch at normotensive strain induces $NO^{\bullet}$ production, whereas hypertensive strain promotes H_2O_2 production, possibly through NADPH oxidase. This sensor revealed new insight to the redox response of endothelial cells under different mechanical stressors.

Figure 4



Microfluidic platform integrating four parallel channels for H_2O_2, $ONOO^-$, $NO^{\cdot}$, and NO_2^- measurements (with permission from the study by Li et al. [65]).

Because oxidative stress is implicated in the progression of many pathological conditions, considerable effort has been made to affect ROS levels under various disease settings for therapeutic benefit. Insights into the actions of established and novel therapeutics were recently addressed by electrochemical methods. Jiang et al. [67] created nanowire electrodes capable of quantifying, at the subcellular level, ROS production in fibroblast and cancer cell lines. This electrode identified the mitochondria, specifically complex IV, as the principal site of ROS production in response to chemotherapeutic. Higher levels of paclitaxel-induced ROS were detected in cancer cells compared with normal cells, suggestive of a selective cytopathic mechanism. Vaneev et al. [69] used platinized nanoelectrodes to demonstrate rapid H_2O_2 evolution in single cells after treatment with chemotherapeutics. The translational utility of this sensor was demonstrated in tumour-bearing mice treated with doxorubicin. In this application, ROS levels increased with increasing tumour depth, highlighting possible spatial heterogeneity within the tumour. Lastly, Gubernatorova et al. [70] evaluated the in vivo ROS scavenging ability of Europium-doped ceria NPs using a Cyt C-based electrochemical biosensor [52]. This study linked O_2^- formation with the induction of inflammatory cytokines during intestinal ischemia-reperfusion injury. Ultimately, a greater understanding of the mechanism by which (chemo)therapeutics exert their effects may facilitate the screening of new drugs that are based on redox dependence, while avoiding interference of redox signalling in normal cells.

Despite the desirable characteristics of electrochemical sensors for *in vivo* ROS monitoring, several technical challenges remain before these sensors realize clinical utility. Biofouling, or adsorption of biomolecules onto the probe, can reduce the sensor's detection capability. This was observed with a carbon nanofiber sensor that initially showed sensitive detection of O_2^- in the rat brain, but sensitivity was reduced by ~60% after implantation [41]. Antifouling strategies that mitigate signal reduction are necessary. Interference by electroactive compounds poses another challenge for *in vivo* use of electrochemical sensors. This was recently addressed by electrodeposition of 1,3-phenylenediamine onto an electrode surface to create a perm-selective barrier. This modification allowed for specific measurement of H_2O_2 flux in the brain [30]. Notwithstanding these challenges, there is an increasing need to accurately measure oxidative status in the clinical setting. The recent COVID-19 pandemic demonstrated the need for platforms that have rapid response time to test clinical specimens. An electrochemical sensor that detects H_2O_2 was developed to screen human sputum for lung inflammation [71]. Reactive oxygen species measurements showed good agreement with the computed tomography scan of the lungs, and infection status could be inferred from the applied potential sweep data. These recent studies highlight electrochemical detection of ROS as a powerful tool for mechanistic and translational studies, but also revealed challenges that are currently being addressed.

Future challenges and trends

Although electrochemical sensors for ROS/RNS monitoring are well-established, most reported work measures concentrations of reactive species in standard solutions or synthetically generated radicals with few reports of implementation in live tissues. Advances in electrode design, featuring increased sensitivity and real-time capabilities, provide a solid foundation for future implementation in biological systems. Because

ROS/RNS is fundamental to many processes and diseases, electrochemical sensors have great potential to facilitate an understanding of their production and removal in cells and tissues, establish the relation between free radical production and disease progression, and evaluate oxidative stress mechanisms. The challenge is to design robust probes and surface modifications that can maintain performance in complex biological environments without passivation or biofouling. While these methods have improved in recent years, their use for rutine applications requires further refinement to address issues such as robustness, selectivity toward specific ROS/RNS, and cross-reactivity. Improving the selectivity toward individual radicals or developing ratiometric or multi-array sensors for simultaneous quantification of a broader range of radicals through parallel measurements is of particular interest for future research. Manufacturing of more robust and stable microelectrodes and biosensors using methods that enable large-scale production is also needed.

Most measurements have been done to study released kinetics in isolated cultures or cells, with few examples of implementation in tissues and organs. Adoption of electrochemical probes to address relevant pathological events relies on interdisciplinary research and close collaboration between electrochemists, biologists, immunologists, and medical doctors. Given the maturity of these probes, future research is expected to explore the use of this technology in relevant cellular and animal models through implantation. An immediate use of implantable microelectrodes is for monitoring ROS/RNS species in real time to better understand their interplay in the biological environment. Innovations in electrode design to increase biocompatibility is also expected. To improve the capabilities of electrochemical measurements, the following potential directions for future research are expected: 1) increasing the sensitivity through improving the electrochemical interface and immobilization strategy by using two-dimensional and three-dimensional nanostructures materials like MXenes, metal−organic frameworks, perovskites, or multilayered polymer layers; 2) scalable manufacturing of microelectrodes to enable large scale adoption and improve reproducibility through the use of additive manufacturing techniques such as printing; 3) multiplexed detection of different ROS/RNS species simultaneously placed along with sentinel or self-reference electrodes to improve accuracy of measurements and minimize interfering effects from coexisting spices; 4) electrode coatings to minimize the non-specific interaction and biofouling effects in biological environments; 5) integration of electrochemical measurements with chemometrics analysis, machine learning and artificial intelligence, as well as wireless connectivity to improve data processing and remote monitoring capabilities of electrochemical measurements; 6) finally, *in vivo* studies with implanted microelectrodes should be validated with suitable biological manipulations to demonstrate usefulness and accuracy of measurements. Monitoring physiological and pathological events such as cancer, ischemia/reperfusion, traumatic brain injury, trauma, and hypovolemic shock, are all relevant models for future applications.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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** of outstanding interest

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