University of South Bohemia in České Budějovice
Faculty of Science

Reactive oxygen species in the biological system: challenges and methodological advances

HABILITATION THESIS

MSc. Ankush Prasad, Ph.D.
2021

Jihočeská univerzita v Českých Budějovicích
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Reaktivní formy kyslíku v biologickém systému: výzvy a metodologické pokroky

HABILITAČNÍ PRÁCE

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Abstract

Oxidative processes during metabolic reactions such as respiration, photosynthesis or oxidative burst are known to be associated with the formation of reactive oxygen and nitrogen species (ROS/RNS). Reactive oxygen species plays an essential role in promoting cellular function and are known to be involved in the regulation of cell signalling cascades. In conditions where the ROS production exceeds the antioxidant capacity, oxidation of polyunsaturated fatty acids, amino acids and nucleic acids is initiated which can ultimately lead to cell death. Within a cellular environment, the generation of ROS is tightly regulated both spatially and temporally. Identification of target biomolecules or specific residues that undergoes radical formation and their subcellular locations are highly essential to understand the role of ROS in health and disease. The presented thesis will provide an overview of the recent trends in free radical research in biology and medicine; our contribution to the understanding of ROS formation and localization within a living system; ROS mediated reactive intermediate formation and its use as a potential oxidative stress marker in both plant and animal models. To understand the nature and reactivity, precise measurement of reactive compounds is required while tools available at the moment are limited by low sensitivity and specificity. In recent times, electrochemical detection and monitoring of ROS has been extensively studied owing to their advantages like quick response time, cost-effectiveness, enhanced sensitivity, and specificity. Thus, the study presented here emphasize recent development on in-vivo detection and visualization of oxidative stress using various techniques and add our contribution to the development of an electrochemical biosensor based on catalytic amperometry to simultaneously monitor oxygen consumption and hydrogen peroxide production in real-time.

Keywords	reactive oxygen species; free radicals; oxidative stress; fluorescent probes; electrochemical biosensors; Arabidopsis, Chlamydomonas; monocytes; confocal laser scanning microscopy; EPR spectroscopy.
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Abstrakt

O oxidačních procesech během metabolických reakcí, jako je dýchání, fotosyntéza nebo oxidační vzplanutí, je známo, že jsou spojeny s tvorbou reaktivních forem kyslíku a dusíku (ROS/RNS). Reaktivní formy kyslíku hrají zásadní roli při podpoře buněčných funkcí a je známo, že se podílejí na regulaci buněčných signálních kaskád. V podmínkách, kde produkce ROS přesahuje antioxidační kapacitu, je zahájena oxidace polynenasycených mastných kyselin, aminokyselin a nukleových kyselin, což může v konečném důsledku vést k buněčné smrti. V buněčném prostředí je generace ROS přísně regulována jak prostorově, tak časově. Identifikace cílových biomolekul nebo specifických zbytků, které odpovídají tvorbě radikálů a jejich umístění ve vnitrobuněčných oddílech, je velmi důležitá pro pochopení role ROS ve zdraví a nemoci. Předložená práce poskytne přehled o nejnovějších trendech výzkumu volných radikálů v biologii a medicíně; náš příspěvek k pochopení tvorby a lokalizace ROS v živém systému; tvorba reaktivního meziproduktu zprostředkovaná ROS a jeho použití jako potenciálního markeru oxidačního stresu v rostlinných i zvířecích modelech. Abychom porozuměli povaze a reaktivitě, je nutné přesné měření reaktivních sloučenin, zatímco nástroje, které jsou v současné době k dispozici, jsou omezeny nízkou citlivostí a specificitou. V nedávné době byla rozsáhle studována elektrochemická detekce a monitorování ROS kvůli jejich výhodám, jako je rychlá doba odezvy, hospodárnost, zvýšená citlivost a specifičnost. Zde uvedená studie tedy zdůrazňuje nedávný vývoj v oblasti in vivo detekce a vizualizace oxidačního stresu pomocí různých technik a přidává náš příspěvek k vývoji elektrochemického biosenzoru založeného na katalytické amperometrii pro současné sledování spotřeby kyslíku a produkce peroxidu vodíku v reálném čase.

Klíčová slova	reaktivní formy kyslíku; volné radikály; oxidační stres; fluorescenční sondy; elektrochemické biosenzory; Arabidopsis, Chlamydomonas; monocyty; konfokální laserová skenovaci mikroskopie; EPR spektroskopie.
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Contents

Attached publications

Prasad A, Kumar A, Suzuki M, Kikuchi H, Sugai T, Kobayashi M, Pospíšil P, Tada M, Kasai S. Detection of hydrogen peroxide in photosystem II (PSII) using catalytic amperometric biosensor. *Frontiers in Plant Science* 6(862): 1-10 (2015).

Prasad A, Ferretti U, Sedlářová M, Pospíšil P. Singlet oxygen production in *Chlamydomonas reinhardtii* under heat stress. *Scientific Reports* 6: 20094 (2016a).

Kikuchi H, **Prasad A**, Matsuoka R, Aoyagi S, Matsue T, Kasai S. Scanning electrochemical microscopy imaging during respiratory burst in human cell. *Frontiers in Physiology* 7(25): 1-6 (2016b).

Prasad A, Kikuchi H, Inoue KY, Suzuki M, Sugiura Y, Sugai T, Tomonori A, Tada M, Kobayashi M, Matsue T, Kasai S. Simultaneous real-time monitoring of oxygen consumption and hydrogen peroxide production in cells using our newly developed chip-type biosensor device. *Frontiers in Physiology* 7: 109 (2016c).

Prasad A, Sedlářová M, Kale RS, Pospíšil P. Lipoxygenase in singlet oxygen generation as a response to wounding: *in vivo* imaging in *Arabidopsis thaliana*. *Scientific Reports* 7: 9831 (2017a).

Prasad A, Kumar A, Matsuoka R, Takahashi A, Fujii R, Sugiura Y, Kikuchi H, Aoyagi S, Aikawa T, Kondo T, Yuasa M, Pospíšil P, Kasai S. Real-time monitoring of superoxide anion radical generation in response to wounding: electrochemical study. *Peer J* 5: e3050 (2017b).

Prasad A, Balukova A, Pospíšil P. Triplet excited carbonyls and singlet oxygen formation during oxidative radical reaction in skin. *Frontiers in Physiology* 9: 1109 (2018a).

Prasad A, Sedlářová M, Pospíšil P. Singlet oxygen imaging using fluorescent probe singlet oxygen sensor green in photosynthetic organisms. *Scientific Reports* 8: 13685 (2018b).

Prasad A, Sedlářová M, Balukova A, Ovsii A, Rác M, Křupka M, Kasai S, Pospíšil P. Reactive oxygen species imaging in U937 cells. *Frontiers in Physiology* 11: 552569 (2020a).

Prasad A, Sedlářová M, Balukova A, Rác M, Pospíšil P. Reactive Oxygen Species as a response to wounding: *in vivo* imaging in *Arabidopsis thaliana*. *Frontiers in Plant Science* 10: 1660 (2020b).

Kumar A, **Prasad A**, Sedlářová M, Ksas B, Havaux M, Pospíšil P. Interplay between antioxidants in response to photooxidative stress in Arabidopsis. *Free radical Biology and Medicine* 160: 894-907 (2020c).

Kumar A, **Prasad A**, Sedlářová M, Kale R, Frankel LK, Sallans L, Bricker TM, Pospíšil P. Tocopherol controls D1 amino acid oxidation by oxygen radicals in photosystem II. *Proceedings of the National Academy of Sciences of the United States of America* 118(4): e2019246118 (2021a).

Prasad A, Manoharan RR, Sedlářová M, Pospíšil P. Free radical-mediated protein radical formation in differentiating monocytes. *International Journal of Molecular Sciences* 22(18): 9963 (2021b).

Overview of the thesis

This habilitation thesis summarizes my research work focused on reactive oxygen species which has been supported by thirteen publications. The first part (Chapters 1-3) deals with reactive oxygen species (ROS) generation in various living systems for which site of ROS production and mechanism of action were identified and validated using different methodological approaches. The studies were carried out on different biological models ranging from cyanobacteria, and individual cells (algae, cancer cell lines) to higher plant tissues. Eight research papers are presented within this topic as listed below in chronological order of publication*:

1. **Prasad A**, Ferretti U, Sedlářová M, Pospíšil P. Singlet oxygen production in *Chlamydomonas reinhardtii* under heat stress. *Scientific Reports* 6, 20094 (2016a).
2. **Prasad A**, Sedlářová M, Kale RS, Pospíšil P. Lipoxygenase in singlet oxygen generation as a response to wounding: *in vivo* imaging in *Arabidopsis thaliana*. *Scientific Reports* 7, 9831 (2017a).
3. **Prasad A**, Balukova A, Pospíšil P. Triplet excited carbonyls and singlet oxygen formation during oxidative radical reaction in skin. *Frontiers in Physiology* 9: 1109 (2018a).
4. **Prasad A**, Sedlářová M, Balukova A, Ovsii A, Rác M, Křupka M, Kasai S, Pospíšil P. Reactive oxygen species imaging in U937 cells. *Frontiers in Physiology* 11: 552569 (2020a).
5. **Prasad A**, Sedlářová M, Balukova A, Rác M, Pospíšil P. Reactive oxygen species as a response to wounding: *in vivo* imaging in *Arabidopsis thaliana*. *Frontiers in Plant Science* 10: 1660 (2020b).
6. Kumar A, **Prasad A**, Sedlářová M, Ksas B, Havaux M, Pospíšil P. Interplay between antioxidants in response to photooxidative stress in Arabidopsis. *Free radical Biology and Medicine* 160: 894-907 (2020c).
7. Kumar A, **Prasad A**, Sedlářová M, Kale R, Frankel LK, Sallans L, Bricker TM, Pospíšil P. Tocopherol controls D1 amino acid oxidation by oxygen radicals in photosystem II. *Proceedings of the National Academy of Sciences of the United States of America* 118(4), e2019246118 (2021a).
8. **Prasad A**, Manoharan RR, Sedlářová M, Pospíšil P. Free radical-mediated protein radical formation in differentiating monocytes. *International Journal of Molecular Sciences* 22(18): 9963 (2021b).

In the second section of presented habilitation (Chapter 4), the development of biosensors and methods for ROS detection are described, as these were also crucial output of my research work. The section is represented by five publications listed below:

9. **Prasad A**, Kumar A, Suzuki M, Kikuchi H, Sugai T, Kobayashi M, Pospíšil P, Tada M, Kasai S. Detection of hydrogen peroxide in Photosystem II (PSII) using catalytic amperometric biosensor. *Frontiers in Plant Science* 6, 862, 1-10 (2015).

10. Kikuchi H, **Prasad A**, Matsuoka R, Aoyagi S, Matsue T, Kasai S. Scanning electrochemical microscopy imaging during respiratory burst in human cell. *Frontiers in Physiology* 7, 25, 1-6 (2016b).
11. **Prasad A**, Kikuchi H, Inoue KY, Suzuki M, Sugiura Y, Sugai T, Tomonori A, Tada M, Kobayashi M, Matsue T, Kasai S. Simultaneous real-time monitoring of oxygen consumption and hydrogen peroxide production in cells using our newly developed chip-type biosensor device. *Frontiers in Physiology* 7:109 (2016c).
12. **Prasad A**, Kumar A, Matsuoka R, Takahashi A, Fujii R, Sugiura Y, Kikuchi H, Aoyagi S, Aikawa T, Kondo T, Yuasa M, Pospíšil P, Kasai S. Real-time monitoring of superoxide anion radical generation in response to wounding: electrochemical study. *Peer J* 5: e3050 (2017b).
13. **Prasad A**, Sedlářová M, Pospíšil P. Singlet oxygen imaging using fluorescent probe singlet oxygen sensor green in photosynthetic organisms. *Scientific Reports* 8: 13685 (2018b).

* Please note that the above articles are presented in chronological order in the list of publications but are referred in the following sub-section based on methodologies/ model organisms used.

Introduction

Oxidative processes during metabolic reactions such as respiration, photosynthesis or oxidative burst are known to be associated with the formation of reactive oxygen and nitrogen species which are commonly referred to as ROS and RNS [1-5]. They are known to play a pivotal role in defense mechanism to combat stress stimuli and participate in signal transduction pathways [2, 3, 6-8]. Reactive oxygen species are well known to be formed either by electron transport from highly reducing species to molecular oxygen (O_2) or by excitation energy transfer from triplet chromophores to O_2. The former leads to the formation of superoxide anion radical ($O_2^{\bullet-}$) which can be further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) [1]. The triplet-singlet energy transfer from the triplet chromophores to O_2 lead to the formation of singlet oxygen ($^1O_2$). The reactivity of the ROS differs; the radical ROS such as $HO^{\bullet}$ are highly reactive due to the presence of unpaired electrons whereas the non-radical species are rather less reactive [1, 9].

The formation of ROS can have a deleterious effect on biomolecules such as lipids, proteins and nucleic acids within the cells which is prevented by the action of non-enzymatic as well as enzymatic systems [10]. The non-enzymatic antioxidant systems consist of low-molecular-weight metabolites such as ascorbate, α-tocopherol, bilirubin, melanin, lipoic acid, trans-resveratrol among others whereas enzymes involved in balancing ROS levels include superoxide dismutase (SOD) consisting of Fe-SOD, Mn-SOD, Ni-SOD, Cu-Zn SOD, and others such as ascorbate peroxidase, glutathione peroxidase, cytochrome c peroxidase and catalases [11-16]. In conditions where the ROS production exceeds the antioxidant capacity, oxidation of polyunsaturated fatty acids, amino acids and nucleic acids is initiated which can ultimately lead to cell death [9, 17, 18].

Identification of target biomolecules or specific residues that undergoes radical formation and their subcellular locations are highly essential to understand the role of ROS in health and disease. Oxidative degradation of molecules which constitute cells, mainly of lipids and proteins, has been subject of extensive research in the past decades [19-22]. In humans, the ageing phenomenon and several lifestyle-related diseases have been linked to ROS generation [23, 24]. Biomolecule-centered radicals pose a short life span and thus can either decay quickly or react resulting in fragmentation, aggregation or posttranslational modifications of biomolecules.

One of the most challenging parts of ROS studies has been detection of individual ROS molecules at the cellular level. In general, ROS detection methodologies described so far can be broadly categorized into direct or indirect methods and their specificity as well as sensitivity has been a matter of intensive discussions. However, with a short lifespan and low concentrations, the direct localization of ROS is possible only for sub-millisecond timescale, except for H_2O_2. Indirect methods involve the reaction of a particular type of ROS with a specific probe or spin trap to yield a stable analyte. Nevertheless, both methodological approaches lack standardized protocols. Techniques such as chemiluminescence have been used as indirect tools for tracking the production of ROS in various organisms but their specific detection had not been possible [25-27]. Electron paramagnetic resonance (EPR) spectroscopy

have been used as a direct technique to detect species such as $HO^{\bullet}$, $^1O_2$ and carbon-centered radicals using spin trapping techniques [28-32]. More lately, fluorescent probes have been used extensively and the development of several new probes was achieved to localize the ROS formation utilizing confocal laser scanning microscopy (syn. confocal microscopy) with tests being performed to check its toxicity, sensitivity to light etc. to avoid artefacts [33-44].

Understanding the mechanism of ROS formation in a living system is extremely crucial in biological research and/or clinical applications. In our recent work presented within the frame of this thesis, I believe to have contributed to the understanding the formation of ROS in the living system, the localization of the ROS formed, and formation of reactive intermediates which can be used as a marker for oxidative stress in both plant and animal models. In addition, I also focused on the development of protocols for *in-vivo* localization and visualization of ROS and reactive intermediates. Last but not least, I contributed to the development of an electrochemical biosensor based on catalytic amperometry that can simultaneously monitor O_2 consumption and production of H_2O_2 in real-time.

Chapter 1. Reactive oxygen species in organisms

During the metabolic process and under the effect of exogenous/endogenous stress factors, ROS are produced in different cell organelles as a consequence of physiology and/or part of defense cellular mechanisms. The source of ROS in cells can be either endogenous or exogenous. The primary endogenous source includes cellular components such as mitochondria, endoplasmic reticulum, and peroxisomes. [1, 45, 46]. The key contributors to ROS generation in mitochondria are complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) which catalyze production of $O_2^{\bullet-}$ which is later converted into H_2O_2 by SOD [47]. In peroxisomes, the electron transfer from various metabolites to O_2 leads to the generation of H_2O_2. Acyl CoA oxidases, L-α-hydroxy oxidase, urate oxidase, D-amino acid oxidase, xanthine oxidase among others present in peroxisomes are known to generate ROS [1, 48]. Besides the major contributors of ROS in cells, the enzymes present in the endoplasmic reticulum are also known to contribute to overall ROS within cellular environment. Exogenous sources have been extensively studied due to environmental changes [49]. It has been observed that stress factors such as oxidizing chemicals, higher ultraviolet (UV) light, ionizing radiations, chemical pollutants, use of pesticides, industrial contaminants, metals etc. induce ROS generations in the living system [50-52].

Some of the ROS formed act as cellular messengers and participate in redox signalling [53]. ROS are regarded as dynamic biological response and mitogenic cues whereas lower concentration is necessary for cellular homeostasis. With its reliable role as second messengers, ROS are produced by nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidases (NOXs) in response to various physiological or chemical stimuli which in turn mediate redox signalling. It has also been known that various growth factors and cytokines stimulate ROS production by activating NADPH oxidase [54-57]. Although it is still not very clear whether ROS produced by mitochondria significantly contribute to signalling, but it is becoming increasingly clear that H_2O_2 released into the cytosol is the key player in a variety

of signalling networks such as cell-cycle transition and balance of redox state [46]. Thus, along with enzymes of respiratory chain, NOXs are recognized as foremost source of ROS within cell.

Using various model systems, we have studied the generation of ROS/intermediate products under normal metabolic processes and during various stress factors using different fluorescent probes (Fig. 1). As a result of heat stress, we have demonstrated the formation of $^1O_2$ *in vivo* in unicellular green alga *Chlamydomonas reinhardtii* (Prasad et al., 2016a). We observed that the integral distribution of singlet oxygen sensor green-endoperoxide (SOSG-EP) fluorescence intensity (also referred to as SOSG intensity or SOSG signal) in heated Chlamydomonas cells was enhanced by about six (6) times as compared to non-heated Chlamydomonas cells. It was moreover evident that $^1O_2$ was formed predominantly in chloroplasts as the SOSG fluorescence corresponded with the chlorophyll fluorescence (Prasad et al., 2016a). Based on the scanning of multiple layers of the Chlamydomonas cells, we claimed that pyrenoids and vacuoles also partially contributed to the overall SOSG fluorescence.

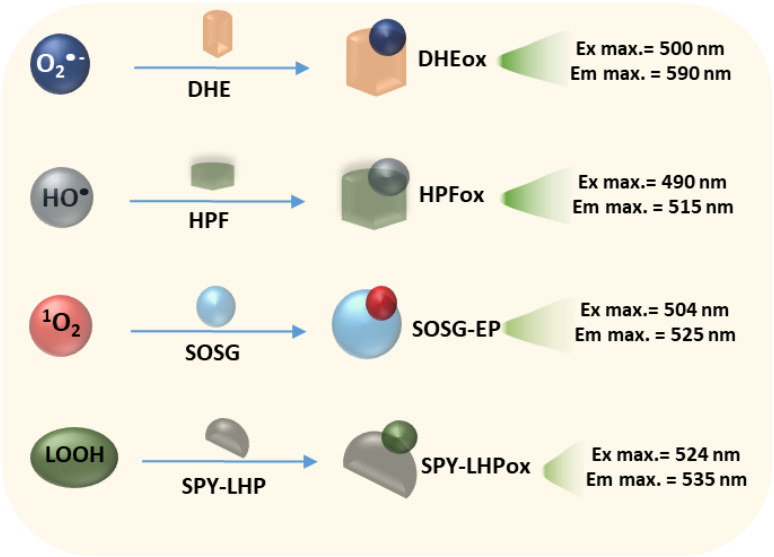

Figure 1: Fluorochrome-based detection of ROS and their spectral properties. (A) Dihydroethidium (DHE) oxidation by $O_2^{\bullet-}$ leading to formation of 2-hydroxyethidium (2-OH-E$^+$) providing fluorescence (excitation/emission maxima of ~500/590 nm). (B) 3′-p-(hydroxyphenyl) fluorescein (HPF) oxidation by HO$^\bullet$ leading to formation of HPFox providing strong green fluorescence (excitation/emission maxima of ~490/515 nm). (C) Singlet oxygen sensor green (SOSG) oxidation by $^1O_2$ leading to SOSG-EP (excitation/emission maxima of ~504/525 nm). (D) Spy-LHP oxidation by LOOH forming Spy-LHPox providing fluorescence (excitation/emission maxima of ~524/535 nm).

The results motivated me to unveil abiotic stresses which induce ROS generation. With this view in mind, we performed experiments with mechanical wounding which is considered among the most intensive stress influencing plants ontogeny and lifetime. Mechanical wounding was tested on a model higher plant, *Arabidopsis thaliana*. Moreover, since ROS has been known to play an indispensable role in the local as well as systemic defense reactions, we measured the formation of ROS immediately after the mechanical wounding. With the usage of ROS probes, we found a significant enhancement in the production of $^1O_2$ similar to that of Chlamydomonas cells using SOSG fluorescence utilizing confocal microscopy. We observed

the highest SOSG fluorescence originating primarily from the cell layers at the cutting edge (Fig. 1, Prasad et al., 2017a, and Prasad et al., 2020b). Number of cell layers with SOSG fluorescence also varied according to the extent of the damage. Based on obtained results, we hypothesized a direct correlation between wounding and the potential role of $^1O_2$ as a signalling messenger. In addition, as the SOSG intensity was higher on the cutting edge and lower on the neighbouring mesophyll cells, this might be an indication that $^1O_2$ might be diffusing to some distance.

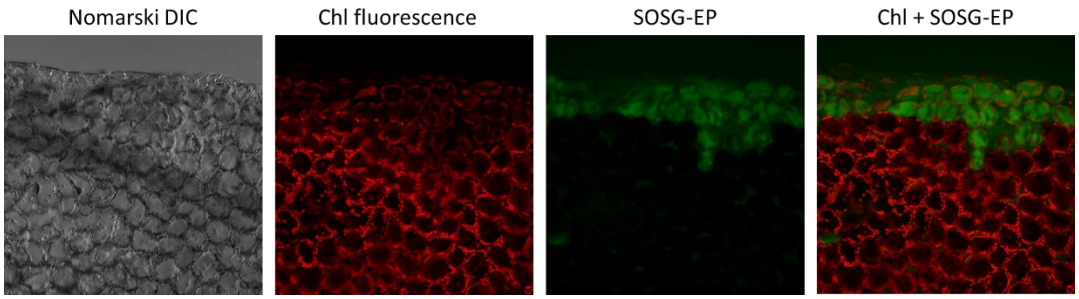

Figure 2: Confocal microscopy imaging of $^1O_2$ as a result of mechanical injury of Arabidopsis leaves. From left to right are Nomarski DIC; chlorophyll fluorescence, SOSG-EP fluorescence and combined channel (chl fluorescence + SOSG-EP) under 20× objective following 30 min of incubation in 50 µM SOSG (E_x/E_m: 488 nm/505–525 nm).

We further performed screening of $O_2^{\bullet-}$ and $HO^{\bullet}$ in high light-induced Arabidopsis leaves (wild type, Col-0 and *vte1* mutant lacking α-tocopherol) using fluorescent probes dihydroethidium (DHE) and 3′-p-(hydroxyphenyl) fluorescein (HPF), respectively. In the dark, low fluorescence was observed in WT. With exposure to high-intensity light, the DHEox and HPFox fluorescence were enhanced in all samples. The DHEox and HPFox fluorescence in *vte1* were significantly higher as compared to WT. Further comparison of Nomarski DIC and DHE or HPF fluorescence channels showed that both ROS were formed predominantly in the chloroplasts. The study led to the conclusion that α-tocopherol is an effective scavenger of $O_2^{\bullet-}$ and $HO^{\bullet}$ in the leaf (Kumar et al., 2020c and Kumar et al., 2021a).

At the tissue level, it was relatively simple to perform the *in-vivo* imaging if we exclude the challenge with the penetration of probes which is discussed in the later sections, we sought to see whether it is promising to detect ROS using the above methodology at a single-cell level. We chose human myeloid leukaemia cell line U937 which bears the potential to differentiate into macrophages or dendritic cells in response to external stimuli. Based on our previous experiences, we used phorbol-12-myristate-13-acetate (PMA) which induces growth arrest and series of morphological changes. The exogenous addition of PMA which is a potent activator of protein kinase C and inducer of ROS production can activate NADPH oxidase and subsequently lead to the formation of $O_2^{\bullet-}$ which upon dismutation can eventually generate H_2O_2 and subsequently $HO^{\bullet}$ [58, 59]. Using HPF, we visualized the formation of $HO^{\bullet}$ and the results indicated that the differentiation and ROS formation are associated phenomena. To my best knowledge, we presented the direct detection of $HO^{\bullet}$ at the single-cell level in human cells for the first time (Prasad et al., 2020a).

This specific topic caught our interest, and we extended the study to several cell lines. We carried out control experiment to check the cell differentiation stages, toxicity of inducers and/or solvents used, membrane integrity etc. using FM4-64 staining (Fig. 2) and screening of ROS (Fig. 3) in different cells under the effect of diverse differentiation inducers. We carried out studies using cell lines U-937, HL-60, and THP-1 and differentiation inducers such as ascorbic acid (AA) and retinoic acid (ATRA) (Fig. 2). As a general statement, we can conclude that in all three model cell lines, ROS formation as a result of cell transition from monocyte to macrophages was evident (Figs. 2, 3 and Prasad et al., 2021b). The duration of treatment of cells with differentiation inducers also influenced the $HO^{\bullet}$ formation. Higher HPFox fluorescence was observed in cells treated with 250nM PMA for 72h compared to 48h (Fig. 4). Doses of inducers and duration of treatments varied in cell lines, for instance, in the case of HL-60, a concentration of 50nM PMA was sufficient for cell differentiation and for visualizing the HPFox fluorescence (Prasad et al., 2021b). For $O_2^{\bullet -}$ visualization in THP-1 cells, incubation with DHE for last 1 h of 24 h differentiated cell was ideal for obtaining DHEox fluorescence (Fig. 4).

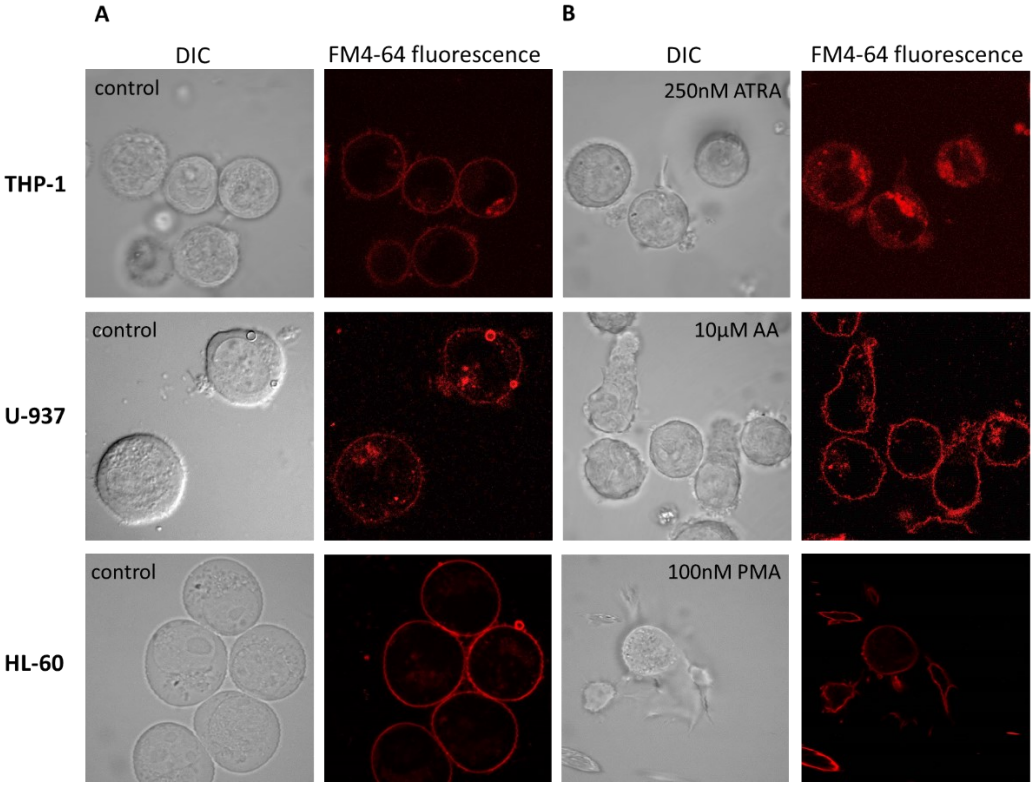

Figure 3: Staining of cell lines (THP-1, U-937 and HL-60) (5 min, 25˚C) to visualize differentiation and membrane integrity in non-differentiated (A) and 48 h differentiated human cells (B). The panels show cells treated with 250 nM retinoic acid (ATRA), 10µM ascorbic acid (AA), and 100 nM PMA. Individual channels for DIC and FM4-64 (red signal) are presented.

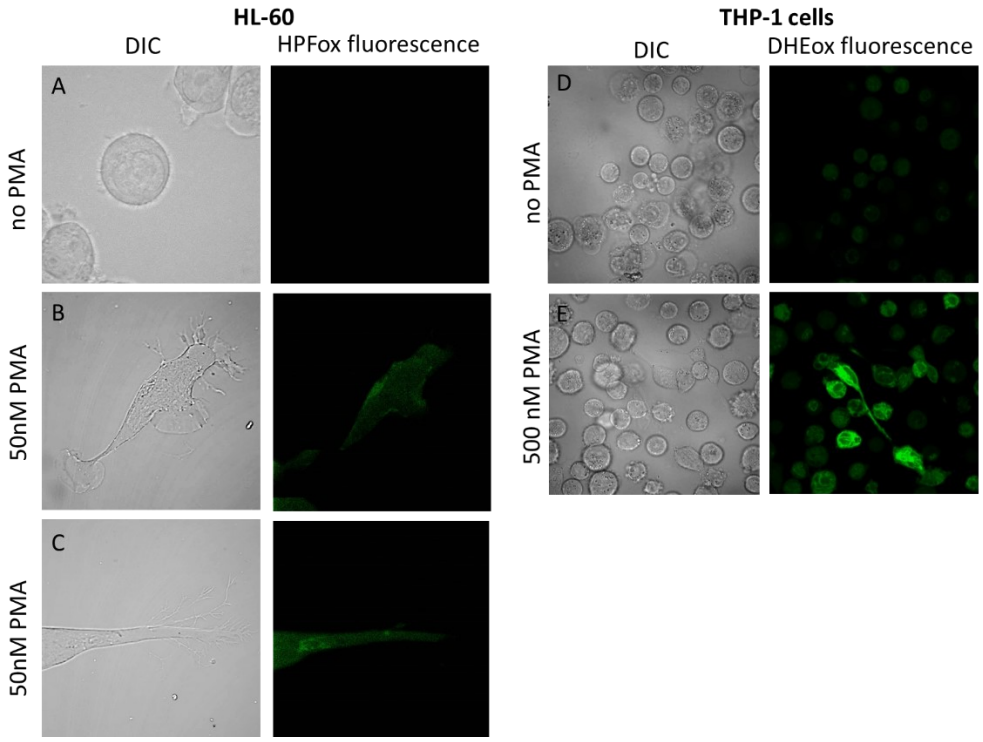

Figure 4: ROS imaging in HL-60 and THP-1 cells. Left panel: the cells were incubated with 1 μM HPF for 12 h in the absence (A) and presence (B, C where C shows magnified image) of 50 nM PMA. The incubation in PMA was done for 48 h. Images from DIC channel and HPFox fluorescence (green signal) is presented. Right panel: the cells were incubated with 1 μM DHE for 1 h in the absence (D) and presence (E) of 500 nM PMA. The incubation in PMA was done for 24 h. Images from DIC channel and DHEox fluorescence (green signal) is presented.

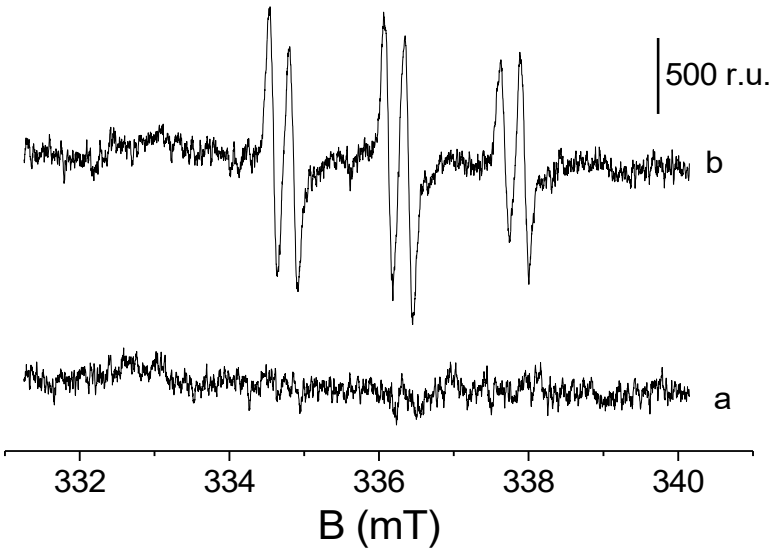

Figure 5: PMA induced POBN-CH(CH_3)-OH adduct EPR spectra. THP-1 cells were incubated with 50 mM POBN in the absence (trace a) or presence of 250 nM ATRA (trace b). Vertical bar represents 500 relative units.

To validate our findings, we performed EPR spectroscopy using spin trapping in THP-1 cells using retinoic acid as a differentiation inducer. The formation of $HO^{\bullet}$ was measured by using a POBN/ethanol spin trap system. Negligible intensity of EPR signal was observed in control THP-1 cells (Figure 5, trace a), whereas differentiated cells (48 h, 250 nM retinoic acid) with 50 mM POBN for 30 min resulted in the formation of the α-hydroxyethyl radical adduct of POBN [POBN-$CH(CH_3)OH$ adduct] EPR signals (Figure 5, trace b).

Chapter 2. Overview of detection techniques

The detection and quantification of ROS have been challenging due to its inherent characteristics such as short life, low physiological concentration, and high reactivity [42, 44, 60, 61]. Before choosing a detection strategy it is important to consider factors like sensitivity of the method, specificity, and selectivity towards analyte under study and the possibility to extend the platform for point of care applications. Added to this, analytical considerations such as robustness, cost effectiveness, availability of necessary instrumentation have largely contributed to development of UV-visible spectroscopic based detection platform. Traditional approaches such as UV-visible spectrometry, chromatography, spectrophotometry, EPR spectroscopy, high-performance liquid chromatography (HPLC) etc. has been used extensively during the past decades, but all the techniques suffer limitations. For instance, EPR spectroscopy discriminates molecules with unpaired electrons by characterizing the electron paramagnetic spectrum and offers higher selectivity. Despite this fact, it has strict requirements in experimental conditions, including but not limited to sample which ideally must be in suspension and its high cost. Although spectrophotometric methods of ROS bears benefit with their simplicity of protocols and low cost, they have a substantially low detection limit as well as sensitivity [62]. Similarly, The HPLC requires a sophisticated instrumentation setup and thus miniaturization ability cannot be achieved which rules out the possibility for routine work.

Distinctive intrinsic properties of ROS like lifetime, generation sources, and diffusion rate can lead to possible inconsistent measurements recorded. Thus, to overcome these limitations associated with traditional detection techniques, sensor-based ROS detection platforms including colorimetric sensor, fluorescent sensor, electrochemical sensor etc. which shows enhanced selectivity and sensitivity compared to the traditional ROS sensing techniques can be employed. Table 1 shows a comparative account of the positive and negative aspects of available ROS detection techniques based on our experiences. Among these, the electrochemical sensor captivated much attention of researchers due to its simplicity in instrumentation and possibilities of real-time kinetic measurement in rather complex biological systems. Electrochemical sensing strategies allow detection of ROS with enhanced sensitivity, lower response time, and possibility of high-resolution plotting of analyte distributions.

Methodology	Specificity	Advantages	Disadvantages
Electron spins resonance spectroscopy	ROS, RNS and reactive intermediates	Specific, sensitive	Expensive, possible neutralization of spin probes
Electrochemical biosensors	$O_2^{\cdot}$-, H_2O_2	Sensitive, user friendly, fast, real time measurements	Prone to electrical noise
Fluorescent probes	$O_2^{\cdot-}$, H_2O_2 , $HO^{\cdot}$, $^1O_2$, intermediate products	Cell permeable, intensity quantifiable, product stable, biocompatibility with biological system	Product complex, low specificity, interfered by $ONOO^-$
Fluorescent proteins	H_2O_2, redox status changes	Dynamic, real-time, cell friendly	Slow in reaction, restriction in receptor cell, less sensitive
Spectrophotometry methods	$O_2^{\cdot-}$, H_2O_2	Sensitive, single product	Low specificity
Chromatography methods	$HO^{\cdot}$	Fast and sensitive	Products complex
Chemiluminescent methods	$O_2^{\cdot-}$, $HO^{\cdot}$	Cell permeable	Low selectivity, intermediates not stable

Table 1: Characteristic features of ROS detection methods.

Electrochemical techniques employ similar principle as the fluorescence technique as it combines electrochemical reactions with specific sensing elements to selectively detect ROS. Very recently, electrochemical biosensors have been employed in studies involving cancerous cells, macrophages, tumorous cells, and plant leaves to detect ROS such as $O_2^{\bullet-}$ and H_2O_2 [63-67]. Most of ROS in biological systems are derived from $O_2^{\bullet-}$ and therefore precise measurement of $O_2^{\bullet-}$ is the most important issue for *in vivo* ROS detection.

Chapter 3. Understanding the mechanism of oxidative radical reactions

With the formation of ROS, abstraction of the weakly bonded hydrogen atom from polyunsaturated fatty acid and amino acid by $HO^{\bullet}$ leads to the formation of lipid and protein alkyl radical ($R^{\bullet}$). In the presence of O_2, the $R^{\bullet}$ reacts with biomolecules leading to the formation of lipid peroxyl radicals and protein peroxyl radicals ($ROO^{\bullet}$). With a further hydrogen abstraction from biomolecule by $ROO^{\bullet}$, lipid or protein hydroperoxide (ROOH) are formed [68]. In the presence of $^1O_2$, via an alternative pathway, ROOH can be formed via the ene reaction (Fig. 6) [69]. Due to the high polarity and lack of unpaired electrons, ROOH bears the capability to migrate from the site of formation. In the presence of transition metal ions such as Fe^{2+}, Mn^{2+}, Cu^{+} or Zn^{+}, the ROOH can be reduced to lipid or protein alkoxyl radical ($RO^{\bullet}$). The formation of organic radicals then leads to the generation of high energy intermediates 1,2-dioxetanes (ROOR) and tetroxides (ROOOOR) [70, 71]. It has also been known that high energy intermediates (ROOR) are formed either by cycloaddition of $^1O_2$ or by the cyclisation of ROO•. Tetroxide, on the other hand, is formed by the recombination of ROO• [72]. In our presented articles, based on the focus of the study, the expressions R, L, and P have been used to indicate biomolecules, lipids, and proteins, respectively.

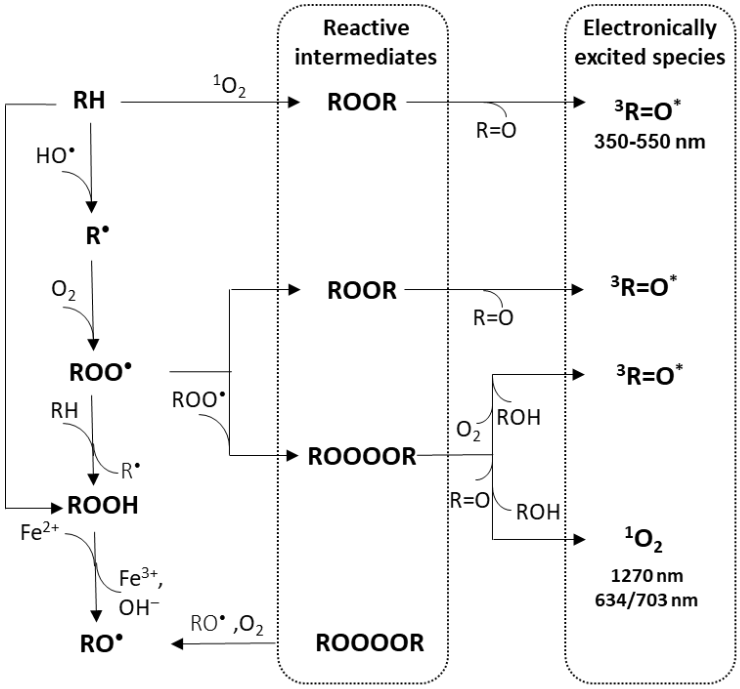

Figure 6: Mechanism of the formation of reactive intermediates and electronically excited species as a consequence of biomolecule oxidation.

We were extremely interested to look at the oxidative process and assess the formation of the reactive intermediates. We applied heat stress which is one of the major environmental stress known to be responsible for lipid peroxidation. In our article Prasad et al., 2016a, we monitored the formation of lipid hydroperoxide (represented by LOOH) in Chlamydomonas cells exposed to 40°C utilizing a probe, Spy-LHP and detected fluorescence by confocal laser scanning microscopy. Spy-LHP is known to form Spy-LHPox after reaction with LOOH [73]. Whereas no evident increase in Spy-LHPox fluorescence was observed in control, pronounced Spy-LHPox fluorescence was observed in the case of Chlamydomonas cells exposed to 40 °C for a period of 30 min (Prasad et al., 2016a). Besides the evident signal coming from chloroplast, small-sized organelles such as vacuoles also showed bright signals. To decipher the pathways and to see whether the process of LOOH generation under light stress is enzymatic or non-enzymatic, we used inhibitors to lipoxygenase (catechol and caffeic acid) which showed suppression in Spy-LHPox fluorescence in heated cells. To validate the findings, we then measured the secondary product of lipid peroxidation, malonaldehyde (MDA) using isocratic reversed-phase HPLC separation of MDA-DNPH adduct which showed a similar pattern (Prasad et al., 2016a).

Taking into consideration that inhibitors might act unspecific due to complex biological systems; we further extended our work and tried to study the role of lipoxygenase in lipid peroxidation. Under the condition of mechanical wounding in Arabidopsis, we compared the formation of triplet carbonyls ($^3L=O^*$, more generally refereed as $^3R=O^*$) which are the final product of lipid peroxidation and acts as a marker for oxidative stress. In Arabidopsis lox2 mutant lacking chloroplast lipoxygenase LOX2, an evident decrease of $^3R=O^*$ was observed compared to WT Arabidopsis. Based on the observations, we concluded that $^3R=O^*$ formation was mediated enzymatically and that lipoxygenase plays a major role in wound-induced $^1O_2$

generation. Based on these findings, we proposed that $^1O_2$ formed during wounding in plants system can be involved in the oxidation of biomolecules which can then take part in plant signalling (Prasad et al., 2017a).

The production of ROS in a biological system under low to moderate stress is in most cases not detectable by the available detection techniques. I was fascinated to understand in-depth the mechanism of lipid peroxidation. In Photosynthetic samples, due to the presence of chlorophyll autofluorescence and its acting as final emitter, it is not always so easy to identify the species formed during the reactions. In view of this point, we chose intact pig ear/skin biopsies as an *ex-vivo*/*in-vitro* model system to represent human skin (Prasad et al., 2018a). Instead of looking for endogenous ROS, we did the exogenous application of Fenton's reagent produced chemically and applied topically on the skin surface. We then monitored the level of electronically excited species generation formed as a consequence of oxidative radical reaction (Fig. 6) by following the photon emission using non-label two-dimensional photon emission imaging and kinetic measurements of ultra-weak photon emission. The results showed that the formation of electronically excited species reflected by total photon emission was directly proportional to the generated ROS. Photon emission was measured using an interference filter type 644 (Schott & Gen., Jena, Germany) with a transmission in the range 340-540 nm and using an Infrared photomultiplier tube (IR-PMT) to determine the contribution of triplet carbonyls ($^3R=O^*$) (Prasad et al., 2018a). It was evident that both electronically excited species ($^3R=O^*$ and $^1O_2$) were formed significantly during the lipid peroxidation (Fig. 6).

Due to the emerging interest in ROS research in relation to human diseases, we were determined to apply the methodologies to animal cell models. Initially, we performed thorough testing on whether the fluorescent probes which are generally used for ROS and intermediate products detection are suitable for animal cell models. Factors like probes half-life, minimum and maximum incubation time, cell penetration capability and cell cytotoxicity were evaluated prior to experimentation. As described in the previous section, we monitored the formation of ROS during differentiation in model cells. Since protein constitutes the majority of cell dry mass, we focused on the oxidative damage to the proteins. The protein damage is initiated by the abstraction of a hydrogen atom by ROS to form protein radicals which can either be carbon-centered or oxygen-centered. With the formation of the transient intermediates, fragmentation, aggregation, or post-translational protein modification occurs [74-76] (Fig. 7). We utilized 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap to stabilize the protein radicals. In order to do so, we added DMPO for the last 24 h of cell differentiation.

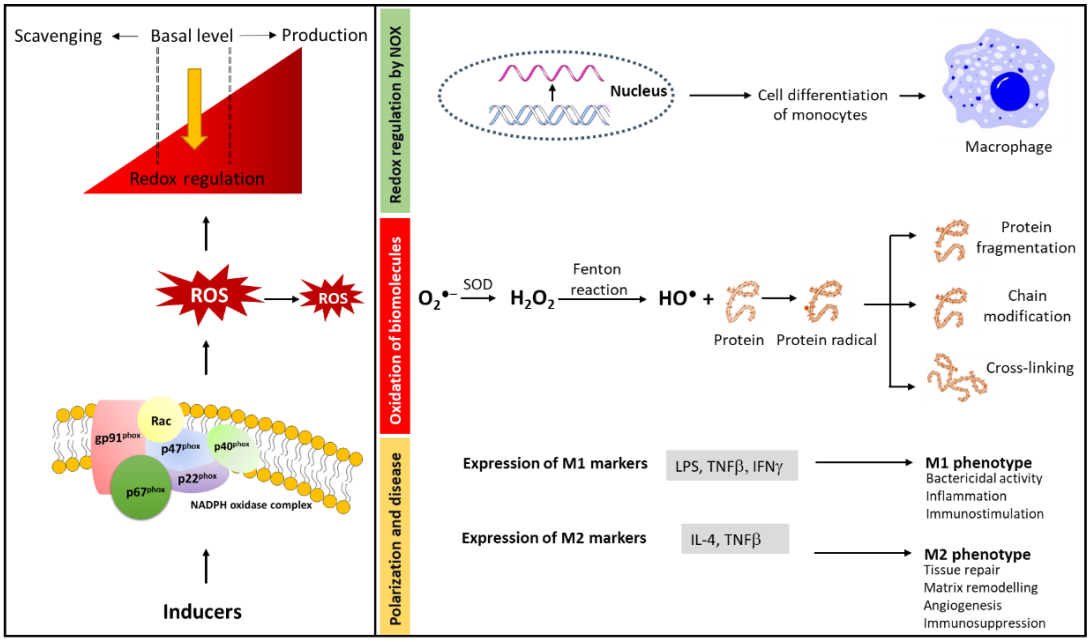

Figure 7: Consequence of ROS generation leading to redox regulation, protein oxidation, and polarization of macrophages and/or disease progression.

Reactive oxygen species-mediated oxidation produces carbonyls groups on protein side chains that serve as a marker for oxidative stress. The free radical-mediated protein oxidation in PMA differentiated U-937 cells was studied using the immuno-spin trapping technique which is based on the trapping of protein radicals with DMPO, forming stable nitrone adducts [77, 78] (Prasad et al., 2021b). Following the homogenization and total protein extraction, we analysed protein radicals using an anti-DMPO antibody by western blot analysis (Prasad et al., 2021b). The results clearly showed prominent NOX4 expression around 67 kDa. As NOXs were noted to be important enzyme involved in ROS production upon stimulation, our study proposed a close relationship between ROS generation and expression of the NOX isoforms. Antibodies specific towards NOX4 were able to pick this protein from whole cell protein preparation and its expression was found to be enhanced with increasing concentration of PMA (150-250nM). To validate the data, NOX specific inhibitor namely apocynin was included along with PMA during experimentation. As expected, expression of NOX4 was found to be downregulated even with increasing concentration of inducer (Prasad et al., 2021b). Apocynin is insufficiently metabolized by PMA stimulated cells and being a pro-drug, it requires activation by myeloperoxidase [79, 80]. In the presence of ROS and myeloperoxidase, apocynin gets activated to form apocynin radical which in turn oxidize thiol in NADPH oxidase. A lag time inhibitory effect of apocynin observed in this study can be attributed to availability of ROS and myeloperoxidase within the intracellular environment.

Chapter 4. Challenges in detection and development of biosensors

Numerous spectroscopic and microscopic methods have been used to detect ROS formation in organisms both *in vitro* and *in vivo*. While *in vitro* detection methods provide information mostly on the quantification of ROS formed, *in vivo* techniques on the contrary provide better indications on the spatiotemporal changes within the cells or tissues. For instance, *in vitro* $^1O_2$ detection such as direct detection of $^1O_2$ by phosphorescence at 1270 nm, chemical trapping based on O_2 uptake owing to the reaction of $^1O_2$ with histidine or imidazole and oxidation of diamagnetic nitroxide to paramagnetic nitroxyl radical detected by EPR spectroscopy is feasible for chemical samples and cell components such as photosystem II (PSII) membranes, thylakoids and chloroplasts but cannot be easily used for *in vivo* plant and animal samples [38, 81-83].

Singlet oxygen sensor green was introduced being one of the best candidates among the fluorescent probes with respect to sensitivity and selectivity. Although it was claimed to be impermeable in most cell compartments, this was later contradicted by authors [38, 84, 85]. Further constraints of the application of SOSG were its light sensitivity leading to photosensitization under UV radiation and visible light [86]. These reports limited its application in any kind of light-induced oxidative stress studies. More detailed study of photochemistry showed that triplet excited SOSG was formed from singlet excited SOSG *via* intersystem crossing which then transfers the energy to O_2 forming $^1O_2$ [87]. This characteristic was a hindrance for SOSG usage, but we decided to re-examine the property. For this purpose, we performed experiments on set of model photosynthetic organisms (cyanobacteria, alga, and higher plant) and summarized the principal factor and conditions under which the probe can be used efficiently (Prasad et al., 2018b). Our results showed that penetration was not a concern in the case of Chlamydomonas cells and uptake of SOSG probe was achieved by incubation of cell in SOSG probe solution. The penetration in Synechocystis cells was restricted because of the presence of polysaccharide matrix-forming mucous surface structures but it is possible to achieve it with mild heating at 37°C which dissolves the mucous sheath. Similarly, to Synechocystis cells, penetration of SOSG to Arabidopsis leaves was also challenging due to the complexity of the tissue comprising the epidermis covered by the cuticle. To overcome this, we used the pressure infiltration using the shut syringe method with caution not to damage the tissue. With the above protocols and based on screening the samples thoroughly with z-scan, we concluded that efficient penetration can be achieved in all the above-mentioned types of samples (Prasad et al., 2018b).

To test the photosensitization of SOSG, we illuminated the probes with either red ($\lambda \geq 600$ nm) or white light (400-750 nm). Exposure of SOSG to dark/red light showed negligible SOSG-EP signal while exposure to white light resulted in marked SOSG-EP fluorescence measured using a fluorescence spectrometer (Prasad et al., 2018b). Study of the photooxidative stress under red light showed enhanced SOSG-EP fluorescence in several studies [88, 89]. This led to the conclusion that SOSG can provide credible results only if used with caution.

Besides $^1O_2$ which is one of the most prevalent ROS in a photosynthetic sample, we also aimed towards the detection of H_2O_2 which is mainly known for its role as a signalling

molecule. In the recent past, several spectroscopic techniques (chemiluminescence and fluorescence) and chromatographic techniques (HPLC coupled with peroxyoxalate chemiluminescence detection) have been used for H_2O_2 determination [90, 91]. For instance, light-induced H_2O_2 have been measured in PSII membranes using the oxidation of thiobenzamide with lactoperoxidase or using AmplexRed fluorescence assays [92-94]. A comparison of H_2O_2 detecting probes such as 3,3′ diaminobenzidine (DAB), Amplex Red (AR), Amplex Ultra Red (AUR) and a europium-tetracycline complex (EuTc) have been compared by infiltrating into leaves of tobacco; it was evident that each probe suffers one or other limitation in terms of toxicity, localization, light sensitivity, and capacity to detect H_2O_2 *in vivo* [33].

Apart from spectroscopic techniques (absorbance, chemiluminescence and fluorescence), an electrochemical method has also been reported for the detection of H_2O_2. In the electrochemical method, the electrode can either be without a mediator or can be mediator based. Among the mediator, methylene blue, hydroquinone, ferrocene, ferrocene carboxylic acid etc. are some of the most used ones [95-98]. In our study, we used Osmium (Os) as a mediator to promote the electrode shuttling between horseradish peroxidase (HRP) and the electrode (Fig. 8, left panel). We fabricated Osmium-HRP (Os-HRP) modified carbon electrode based on the protocol described our article, Prasad et al., 2015. Following the basic characterization using cyclic voltammetry, we presented electrochemical measurement which showed that under the illumination of continuous white light at 1,000µmol photons m^{-2} s^{-1}, PSII membranes (150 µg Chl ml^{-1}) resulted in a change in the reduction current which gradually increased with illumination period. The maximum reduction current of 400 pA was reached after about 15 min of light illumination (Prasad et al., 2015). Very limited application on the use of electrochemical sensors have been demonstrated in plant cells and to my best knowledge, it has been only shown at the level of protoplast under the effect of benzoquinone [99, 100]. Thus, our study on PSII membrane and direct detection of H_2O_2 was for the first time reported.

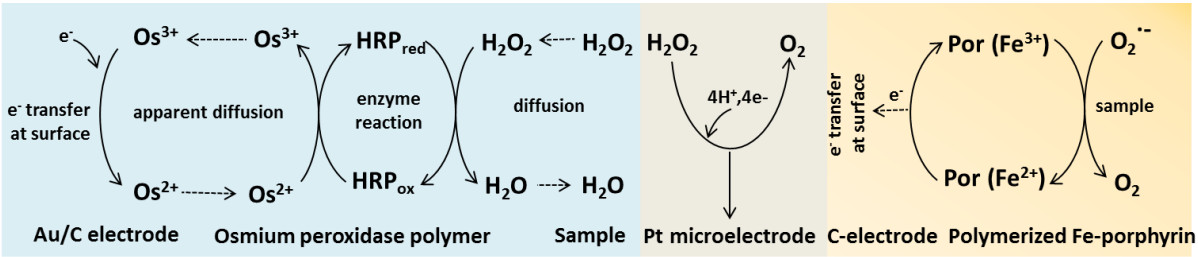

Figure 8: Schematic illustration of the reaction mechanism of the catalytic amperometric method of H_2O_2 detection using Os-HRP polymer (left panel, adopted with modification from Prasad et al., Frontiers in Physiology 2016c); oxygen consumption using platinum microelectrode (middle panel) and $O_2^{\bullet-}$ using iron-porphyrin polymer (right panel).

The formation of ROS is coupled with oxygen consumption and spatiotemporal monitoring of the rapid consumption of oxygen by using scanning electrochemical microscopy (SECM) imaging can also be achieved (Prasad et al., 2016b). We aimed to design and develop a biosensor device that can simultaneously detect the oxygen consumption and production of ROS. In the next study (Prasad et al., 2016c), we produced a chip-type electrochemical device

and tested it on THP-1 cells. The reason behind choosing THP-1 cells was their differentiation to macrophages under the effect of inducers such as PMA. PMA is a potent carcinogenic compound involved in cell growth and differentiation acting *via* protein kinase C (PKC) pathway directly; it is known to cause activation of NADPH oxidase which produces $O_2^{\bullet-}$ and subsequently H_2O_2 and $HO^{\bullet}$ in the cells. We fabricated a chip-type electrochemical biosensor where using photolithography, we created a platinum circuit pattern (diameter, 20 µm; working electrode for detection of oxygen) and gold (Au) pattern (diameter, 1 mm; working electrode for detection of H_2O_2) on a glass substrate. In the following steps, we immobilized the surface of Au electrode with Os-HRP (Prasad et al., 2016c). A polydimethylsiloxane (PDMS) prepolymer with a glass tube was created on top of the circuit to accommodate the cells as represented in Fig. 9. After characterization and calibration of electrodes, we used the chip-type biosensor device (comprising of Pt microelectrode and Os-HPR modified Au electrode) to monitor oxygen reduction current and reduction current for H_2O_2, respectively at -0.5 V vs Ag/AgCl and 0.0 V vs Ag/AgCl. The results showed that under the respiratory burst in THP-1 cell, the reduction currents changed drastically showing a total oxygen consumption of 297mM (in a time span of 110 min) and 494nM H_2O_2 generation (in a time span of 175 min) (Prasad et al., 2016c). As an output of this study, we have developed a catalytic amperometric chip-type electrochemical biosensor device that can detect oxygen consumption and H_2O_2 simultaneously and in real-time (Prasad et al., 2016c). This biosensor chip type device can be used for various biological samples with ease and can also be extended to clinical trials.

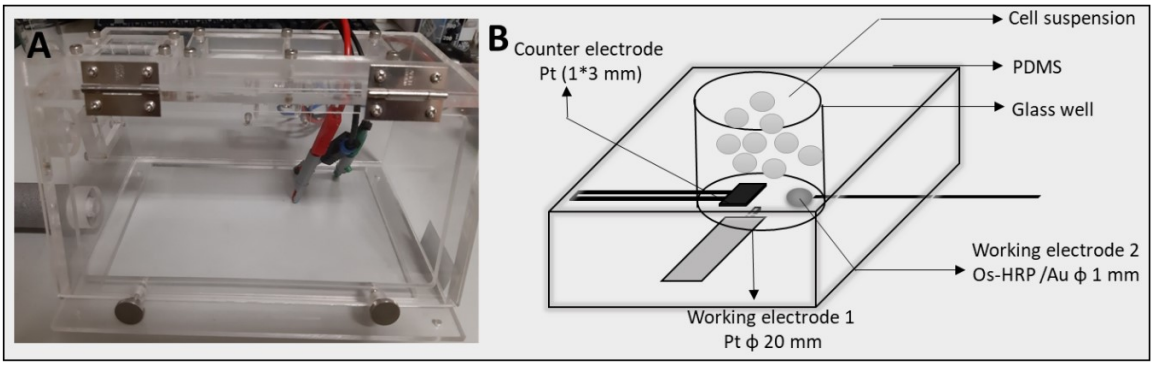

Figure 9: Experimental setup of catalytic amperometry with our newly developed chip-type device. The system depicted in the chamber shows our home-designed incubator (A) for controlled atmosphere (CO_2 and temperature) and the schematic depiction of cell accommodating well (B) consisting of microelectrodes.

For electrochemical detection of ROS, integration of metalloporphyrins into electropolymerized polymer electrodes has been used during the recent past. Thus, in our following study, we took a step forward for the detection of $O_2^{\bullet-}$ by polymeric iron-porphyrin-based modified carbon electrode. The reaction mechanism has been presented in Fig. 8 (right panel). Since we wanted to target *in vivo* or at least *ex-vivo* samples, we positioned the electrode (φ=1 mm) at 1mm above an Arabidopsis leaf with Ag/AgCl as the reference electrode and by using platinum wire as a counter electrode (Prasad et al., 2017b). The electropolymerization step for preparing the working electrode has been described in Prasad et al., 2017b. Following the basic characterization and testing on the chemical system, we performed experiments to

see the effect of wounding and subsequent generation of $O_2^{\bullet-}$. We monitored the oxidation current for $O_2^{\bullet-}$ with different levels of injury and found it evident that $O_2^{\bullet-}$ production increased significantly with extent of mechanical injury. Thus, with the employment of different modified electrodes, it was evident that specific ROS can be detected. The biggest advantage represents the real-time kinetic measurement which is difficult to explore by means of other available methods. Thus, we believe that it could have a strong potential for extensive application in plant research as well.

Summary

Reactive oxygen species formed under physiological conditions in several organelles play a dual role being important for induction of cell division/differentiation and at the same time, playing role in cell ageing and inducing cell death because of oxidation of biomolecules. More recently, researchers have been focusing on the good side of ROS for its activity as signalling molecules. Similar to nitric oxide, ROS is known to regulate and maintain physiological functions predominantly by interacting with cysteine (Cys) residues of proteins. It has been shown that H_2O_2 interacts with Cys thiolate anions ($Cys–S^-$) which oxidizes to their sulfenic form (Cys–SOH) [53, 75, 101, 102]. The redox change then affects transcription, phosphorylation of proteins, and acts in signalling.

In plants, ROS plays role in pathogen defense, programmed cell death and behaviour of stomata [103]. In addition, it is also known to interplay with hormones to regulate the developmental process and stress responses [104]. In Arabidopsis, it has been seen that $O_2^{\bullet-}$ accumulates in the meristematic zone of the root tip and H_2O_2 in the elongation zone for cell division and differentiation, respectively. Once the ratio of $O_2^{\bullet-}$ and H_2O_2 is at a certain level, the transition from cell division to elongation is promoted [105, 106]. Several transcription factors have been studied which acts as key regulators [106-108]. In animal systems, *in vitro* culture models serve as an important tool to understand pathways involved during cell growth, differentiation and disease progression. It has been experimentally confirmed that cells maintain an enhanced level of ROS within the cellular environment in order to proliferate however to level below which will be cytotoxic [109]. In recent times, studies focusing on ROS mediated signalling events in stem cell research were of growing interest wherein dose-dependent activity of ROS are deciphered. ROS production in stem cells is tightly regulated to sustain tissue homeostasis and tissue repair [75]. It is still difficult to precisely understand how different ROS can regulate signalling events and drive the cellular processes. At times, it is also not easy to conclude whether oxidative stress is the cause or a consequence of biomolecule oxidation. It is thus very essential to understand the cross-talk between the ROS levels and the regulatory mechanism involved.

The various methodologies/techniques discussed here can offer insights into redox dynamics, but they have downsides in one way or the other and thus it is crucial to understand them to generate sound experimental design and good results. The fluorescence and electrochemical methods for ROS detection are relatively easy to use with excellent sensitivity and selectivity. Being said that the fluorescence and electrochemistry offer approximately the same sensitivity, the simplicity of the electrochemical method has gained more attention

recently due to real-time detection using microelectrode or even nanoscale electrodes. For choosing the appropriate method or technique for ROS measurement, one must take into consideration the properties of the ROS, its level of production, localization, diffusion, its transformation among different forms etc.

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List of abbreviations

$^1O_2$	singlet oxygen
2-OH-E^+	2-hydroxyethidium
$^3L{=}O^*$	triplet carbonyls
Ag/AgCl	silver/silver chloride
AR	amplex red
AUR	amplex ultra red
Cu-Zn SOD	copper, zinc superoxide dismutase
Cys	cysteine
DHE	dihydroethidium
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DMPO	5, 5-dimethylpyrroline-N-oxide
EPR	electron paramagnetic resonance
Fe-SOD	iron superoxide dismutase
H_2O_2	hydrogen peroxide
$HO^\bullet$	hydroxyl radical
HPF	3′-p-(hydroxyphenyl) fluorescein
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IR-PMT	infrared photomultiplier tube
Spy-LHP	Spy-lipid hydroperoxide
LOOH	lipid hydroperoxide
LOX	lipoxygenase
MAPKs	mitogen activated protein kinases
Mn-SOD	manganese superoxide dismutase
MDA	malonaldehyde
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
Ni-SOD	nickel superoxide dismutase
O_2	molecular oxygen
$O_2^{\bullet-}$	superoxide anion radical
PDMS	polydimethylsiloxane
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PSII	photosystem II
$R^\bullet$	alkyl radical
$ROO^\bullet$	peroxyl radicals
ROS	reactive oxygen species
ROOH	hydroperoxide
ROOR	1, 2-dioxetane
ROOOOR	tetroxide
RNS	reactive nitrogen species
SECM	scanning electrochemical microscopy

SOD	superoxide dismutase
SOSG	singlet oxygen sensor green
SOSG-EP	singlet oxygen sensor green endoperoxide
UV	ultraviolet