INFLUENCE OF VITAMIN C INCORPORATED POLYCAPROLACTONE TOWARDS OXIDATIVE STRESS RELATED RESPONSE IN BONE REGENERATION IN VITRO

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by

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LIST OF SYMBOLS

~	Range
^{0}C	Degree Celsius
et al	et alia
Ø	Diameter
α	Alpha
β	Beta
γ	Gamma

LIST OF ABBREVIATIONS

µL	Micro liter
3-D	Three dimensional
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ARS	Alizarin Red S staining
ATP	Adenosine triphosphate
BMP-2	Bone morphogenetic proteins-2
BMP-7	Bone morphogenetic proteins-7
BMPs	Bone morphogenetic proteins
BTE	Bone tissue engineering
BV	Blood vessels
CaP	Calcium phosphate
COX	Cyclooxygenases
DAPI	4′,6-diamidino-2-phenylindole
DEPMPO	5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide
DCFDA	Dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECGM2	Endothelial Cell Growth Medium 2
ECM	Extracellular matrix
ECs	Endothelial cells
EDS	Energy-dispersive X-ray
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EO	Endochondral ossification
ERK	Extracellular regulated kinases
ESR	Electron spin resonance
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GFs	Growth factors
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HIF-1 α	Hypoxia-inducible factor-1 α
$HO^{\bullet}$	Hydroxyl radicals
Hr	Hours/Hour
HUVEC	Human umbilical vein endothelial cells
IGF-I	Insulin-like growth factor I
JNK	Jun N-terminal kinase
LOX	Lipoxygenases
MAPK	Mitogen-Activated Protein Kinase
Mg	Milligram
Ml	Milliliter
Mm	Millimeter
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
nm	Nanometer
$O_2^{\bullet-}$	Superoxide anion
OBs	Osteoblasts
OCN	Osteocalcin
OCs	Osteoclasts
OD	Optical density
ON	Osteonectin
OPN	Osteopontin
P38	Mitogen-activated protein kinase 38
PBS	Phosphate buffered saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PHD	Prolyl hydroxylase domain
PMA	Phorbol 12-myristate 13-acetate
$PO4^{3-}$	Phosphate
qPCR	Quantitative real-time PCR
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

RANK	Receptor activator of NF-kB
ROS	Reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
Runx-2	Runt-related transcription factor 2
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscope
SIMR	Sharjah Institute for medical research
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TCP	Tricalcium phosphate
TE	Tissue engineering
TGF	Transforming growth factor
TGF-β1	Transforming growth factor beta-1
TNF-α	Tumor necrosis factor-α
TRAP	Tartrate-resistant acidic phosphatase
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
XTT	(2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

LIST OF APPENDICES

PENGARUH POLIKAPROLAKTON TERGABUNG VITAMIN C TERHADAP TINDAK BALAS BERKAITAN TEKANAN OKSIDATIF DALAM PENJANAAN SEMULA TULANG IN VITRO

ABSTRAK

Penjanaan semula tulang kekal sebagai cabaran kritikal dalam sains biobahan kerana penjanaan spesies oksigen reaktif (ROS) yang disebabkan oleh implantasi biobahan semasa proses penyembuhan luka. Tekanan oksidatif berikutan implantasi biobahan boleh menyebabkan keradangan kronik dan menjejaskan integrasi bahan tisu, sekali gus menghalang penyembuhan yang berkesan. Penggabungan antioksidan ke dalam biobahan boleh mengawal tekanan oksidatif dan meningkatkan pertumbuhan semula tulang. Kajian ini menyiasat penggunaan membran polikaprolakton (PCL) yang digabungkan dengan Vitamin C (Vit C) untuk mengurangkan kerosakan akibat ROS dan menjelaskan mekanisme yang meningkatkan proses osteogenik dan angiogenik yang diperlukan untuk penjanaan semula tulang secara in-vitro. Dua jenis membran PCL telah dihasilkan, pertama menggunakan membran PCL 11% berat yang digabungkan dengan 25% berat Vit C (PCL-Vit C) dan yang kedua ialah membran PCL 11% berat sahaja. Kedua-dua membran telah dicirikan menggunakan mikroskop elektron pengimbasan dengan spektroskopi sinar-X penyebaran tenaga (SEM-EDS), spektroskopi inframerah transformasi Fourier (FTIR) dan hidrofilik permukaan. Pelepasan Vit C daripada membran PCL-Vit C dikira secara kolorimetrik. Kajian daya maju dan penempelan sel hFOB 1.19 pada membran telah dijalankan menggunakan ujian XTT, SEM dan mikroskop konfokal. Penjanaan ROS diukur dan pengaruhnya terhadap penanda osteogenik dan angiogenik untuk biomineralisasi telah disiasat menggunakan sel hFOB 1.19 monokultur dalam fasa I, kultur bersama osteoblast-

osteoklas (OB-OC) dalam fasa II dan kultur bersama sel osteoblas+endothelial (hFOB+HUVEC) dalam fasa III kajian melalui ekspresi gen mRNA, ekspresi protein ELISA, kajian pewarnaan mineralisasi, *Western blot* dan laluan isyarat MAPK. Pencirian bahan mendedahkan gentian licin, halus, bebas manik dengan puncak FTIR yang mensimulasikan PCL dalam kedua-dua membran, hidrofilisiti yang lebih tinggi dalam membran PCL-Vit C dan pembebasan Vit C terkawal yang berterusan dalam sejam pertama. Keputusan dalam fasa I menunjukkan PCL-Vit C mempunyai tahap ROS yang lebih rendah berbanding membran PCL dengan lekatan, percambahan, dan pembezaan osteoblas yang lebih baik. Dalam kultur bersama OB-OC fasa II, membran PCL-Vit C meningkatkan penanda osteogenik utama ALP, Col1 dan OCN, mengurangkan nisbah RANKL/OPG, dan pemendapan mineral dipertingkatkan, mencadangkan kesan yang menggalakkan terhadap osteoblastogenesis sambil menghalang osteoklastogenesis. Dalam kultur bersama hFOB+HUVEC fasa III, PCL-Vit C menghasilkan ekspresi HIF-1α yang berkurangan, pengaktifan laluan MAPK dan peningkatan pelepasan VEGF, menggalakkan gandingan angiogenik-osteogenik yang penting untuk neovaskularisasi. Kajian ini menggariskan potensi terapeutik penggabungan antioksidan ke dalam biobahan untuk mengurangkan tekanan oksidatif yang disebabkan oleh ROS, dengan itu mengoptimumkan persekitaran mikro selular untuk penjanaan semula tulang. Penemuan ini menyediakan asas asas untuk pembangunan biobahan pintar yang mengawal selia ROS yang menyokong proses osteogenik dan angiogenik untuk penjanaan semula tulang.

INFLUENCE OF VITAMIN C INCORPORATED POLYCAPROLACTONE TOWARDS OXIDATIVE STRESS RELATED RESPONSE IN BONE REGENERATION IN VITRO

ABSTRACT

Bone regeneration remains a critical challenge in biomaterial science due to reactive oxygen species (ROS) generation induced by biomaterial implantation during the wound healing process. Oxidative stress following biomaterial implantation can lead to chronic inflammation and impair tissue-material integration, thus hindering effective healing. Incorporation of antioxidants into biomaterials may control oxidative stress and enhance bone regeneration. This study investigated the use of polycaprolactone (PCL) membrane incorporated with Vitamin C (Vit C) to mitigate ROS-mediated damage and elucidate the mechanisms that enhance the osteogenic and angiogenic processes required for bone regeneration *in-vitro*. Two types of PCL membrane were produced, first using 11 wt% PCL membrane incorporated with 25 wt% Vit C (PCL-Vit C) and the second was 11 wt% PCL membrane alone. Both membranes were characterized using scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDS), Fourier-transform infrared spectroscopy (FTIR) and surface hydrophilicity. Vit C release from PCL-Vit C membrane was quantified colorimetrically. Viability and attachment studies of hFOB 1.19 cells on membranes were carried out using XTT assay, SEM and confocal microscopy. ROS generation was measured and its influence on osteogenic and angiogenic markers for biomineralization was investigated using monoculture hFOB 1.19 cells in phase I, co-culture of osteoblast-osteoclast (OB-OC) in phase II and co-culture of osteoblast+endothelial cells (hFOB+HUVEC) in phase III of the study; through

mRNA gene expressions, ELISA protein expressions, mineralization staining studies, western blotting and MAPK signalling pathways. Material characterization revealed smooth, fine, bead-free fibres with FTIR peaks simulating PCL in both membranes, higher hydrophilicity in PCL-Vit C membrane and sustained controlled release of Vit C in the first hour. Results in phase I showed PCL-Vit C had lower ROS levels compared to PCL membrane with improved osteoblast adhesion, proliferation, and differentiation. In phase II OB-OC co-cultures, PCL-Vit C membrane enhanced key osteogenic markers ALP, Col1 and OCN, reduced the RANKL/ OPG ratio, and enhanced mineral deposition, suggesting a favorable impact on osteoblastogenesis while inhibiting osteoclastogenesis. In phase III hFOB+HUVEC co-cultures, PCL-Vit C resulted in reduced Hypoxia-inducible factor 1-alpha (HIF-1α) expression, activation of MAPK pathways and increased VEGF release, promoted angiogenic-osteogenic coupling which is critical for neovascularization. This study underscores the therapeutic potential of antioxidant incorporation into biomaterials to mitigate ROS-induced oxidative stress, thus optimizing cellular microenvironments for bone regeneration. These findings provided a foundational basis for the development of ROS-regulating smart biomaterials that supported osteogenic and angiogenic processes for bone regeneration.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Aerobic respiration marks a significant evolutionary advancement, allowing organisms to efficiently extract energy from complex organic molecules through an electron transport chain where oxygen serves as the final electron acceptor (Bedard & Krause, 2007). However, it introduces a notable challenge: the production of reactive oxygen species (ROS), including superoxide anion (O_2•−), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO•) (D'Autréaux & Toledano, 2007). These ROS are generated as by-products of cellular metabolism via lipoxygenases (LOX) and cyclooxygenases (COX) in mitochondria, and can also be produced by endothelial and inflammatory cells (Al-Gubory *et al.*, 2012). Although mitochondria have innate mechanisms to scavenge ROS, such as the production of antioxidant enzymes, these mechanisms are often insufficient to handle the ROS levels generated. Consequently, additional cellular defense strategies are necessary (Glasauer & Chandel, 2014) therefore, maintaining a balance between their beneficial and harmful effects becomes essential.

The generation of ROS, primarily during cellular respiration, can lead to significant cellular damage if not properly regulated. Excess ROS can affect cellular structures such as proteins, lipids, and nucleic acids (Wu *et al.*, 2013; Zhang *et al.*, 2016). While ROS are involved in important cellular functions such as protein phosphorylation, activation of transcription factors, apoptosis, immune responses, and cell differentiation, their levels must be controlled to prevent oxidative damage (Rajendran *et al.*, 2014). An overload of ROS is associated with oxidative stress, which is linked to the onset and progression of various diseases, including cancer, diabetes,

and cardiovascular conditions (Taniyama & Griendling, 2003) and failure of dental and orthopaedic implants (Mouthuy *et al.*, 2016).

In the absence of oxygen, cells resort to anaerobic respiration, a less efficient metabolic process for adenosine triphosphate (ATP) production. During anaerobic respiration, glucose is metabolized into lactic acid (in animals) or ethanol and carbon dioxide (in yeast and some bacteria), resulting in less ATP production compared to aerobic respiration (Saltveit, 2019). This shift to anaerobic respiration, which occurs under oxygen-limited conditions, underscores the crucial role of oxygen in efficient energy production. Without oxygen, cells may experience increased cellular stress due to the accumulation of metabolic byproducts like lactic acid, further complicating energy availability and suppress tissue regeneration (Storey & Storey, 2004).

The challenge of tissue regeneration becomes evident in the context of injuries, infections, cancers, and degenerative diseases. Minor tissue defects may heal spontaneously, but larger critical size defects often require biomaterial scaffolds for effective regeneration. When biomaterials are implanted into bone tissue, it may induce ROS generation. While ROS are necessary for cellular signalling and metabolism, excessive ROS production can overwhelm the endogenous antioxidant defenses, leading to oxidative stress and impairing tissue regeneration and wound healing (Lee *et al.*, 2021).

Bone tissue, characterized by its continuous remodelling through bone resorption and formation, is particularly affected by redox imbalance when oxidants surpass antioxidant activity. During bone remodelling process, bone turnover markers (BTMs) are released into circulation, with bone resorption occurring rapidly within about 10 days and bone formation taking approximately 3 months to complete. The

faster rate of osteoclastic activity compared to osteoblastic activity presents challenges for effective bone and skeletal repair and regeneration.

Several bone cells play a critical role in bone remodelling and osteogenesis. Osteoblasts (OBs) are derived from multipotent Mesenchymal stem cells (MSCs) and account for approximately 4–6% of the bone cell. These mature cuboidal cells are located on bone surfaces and have a lifespan ranging from a few days to around 100 days. The differentiation of MSCs into OBs is initiated by growth factors such as Wnt proteins and bone morphogenetic proteins (BMPs), which are essential for lineage commitment. Subsequently, several genes, including *Runt-related transcription factor 2 (Runx2), Distal-less homeobox 5 (Dlx5),* and *Osterix (Osx),* are expressed, with *Runx2* regulating the expression of key osteoblastic markers such as *collagen type 1 alpha 1 (Col1a1), alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN)* (Florencio-Silva *et al.*, 2015). Osteocytes, the most abundant bone cells, make up approximately 95% of the bone cell population, and this proportion increases with the age and size of bone (Niedzwiedzki & Filipowska, 2015). These osteocytes regulate bone formation and resorption by secreting factors such as sclerostin.

Overproduction of ROS negatively impacts osteoblasts by impairing their differentiation and function. A study by Liu *et al.* (2004) demonstrated that oxidative stress suppresses osteoblast differentiation, as there was a significant reduction in alkaline phosphatase (ALP) activity. Subsequent research further revealed that oxidative stress induced by hydrogen peroxide (H_2O_2) inhibits additional markers of osteoblast differentiation, including the phosphorylation of the transcription factor Runx2 and the formation of osteoprogenitors proteins (Bai *et al.*, 2004). The role of ROS in this inhibition was confirmed when metallothionein, a ROS production inhibitor, restored osteoblast differentiation (Liu *et al.*, 2004). In addition, the

suppression of osteoblast activity under oxidative stress is primarily mediated through the ERK and NF-κB signaling pathways (Bai *et al.*, 2004). Moreover, excessive ROS have been shown to impede bone formation, particularly during the mineralization phase (Arai *et al.*, 2007), and may also contribute to osteoblast apoptosis via the Wnt/β-catenin signaling pathway (Manolagas & Almeida, 2007).

Osteoclasts (OCs), on the other hand, originate from hematopoietic cells of the mononuclear lineage and are tasked with the resorption of bone matrix. Together, OBs and OCs along with the blood supply are the key players in the continuous process of bone remodeling, which persists throughout life. This remodeling cycle includes three interrelated phases: resorption, reversal, and formation, each of which is critical to maintaining skeletal integrity. The overactivity of OCs can lead to bone-degenerative conditions such as osteoporosis and osteolytic bone metastases, while their underactivity is associated with conditions like osteopetrosis (Oikawa *et al.*, 2013). OC differentiation is primarily driven by two growth factors: macrophage colony-stimulating factor (M-CSF), produced by osteoprogenitor mesenchymal cells and OBs, and receptor activator of nuclear factor kappa-B ligand (RANKL), which is expressed by OBs, osteocytes, lymphocytes, and stromal cells. The production of these factors is further stimulated by various systemic and local signals, including parathyroid hormone (PTH), vitamin D, interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) (Beauvais *et al.*, 2016).

ROS play a dual role in bone resorption, being essential for normal osteoclast function while also exerting negative effects on bone metabolism when excessively elevated (Wauquier *et al.*, 2009). During the differentiation of monocytes into osteoclasts, ROS act as critical secondary messengers in RANKL-mediated signaling pathways that drive the expression of NFATc1, a key transcription factor (Callaway &

Jiang, 2015). In addition, the bone resorption process involves cathepsin K-mediated degradation of tartrate-resistant acid phosphatase (TRAP), which, in turn, enhances ROS production to facilitate the final stages of matrix breakdown (Vääräniemi *et al.*, 2004). Hypoxia-induced ROS accumulation may further accelerate the breakdown of the organic matrix, as prolonged stabilization of hypoxia-inducible factor 1-alpha (HIF-1α) has been associated with increased ROS levels and oxidative stress, thereby promoting osteoclast activation (Bell *et al.*, 2011). Moreover, RANKL has been shown to suppress FoxO transcription factor activity, leading to reduced expression of antioxidant enzymes such as catalase, which normally mitigates ROS, including hydrogen peroxide (H_2O_2), further exacerbating oxidative stress (Bartell *et al.*, 2014).

ROS-mediated angiogenesis is closely correlated to oxidative stress. Low levels of ROS can promote healthy blood vessel growth and formation while excessive ROS can cause oxidative stress, damaging tissues and contributing to pathological conditions like cancer and chronic diseases (Huang & Nan, 2019). The interaction between oxidative stress and angiogenesis is centred on the vascular endothelial growth factor (VEGF) signalling pathway (Li *et al.*, 2010). VEGF promotes ROS production by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in endothelial cells.

Despite the body's endogenous antioxidant defenses, signalling pathways like Nrf2 can modulate oxidative stress during tissue regeneration. Oxidative stress from injury, bacterial toxins, or bone augmentation procedures can hinder the bone regeneration process.

Sources of antioxidants that are used to mitigate and scavenge ROS are endogenous antioxidants such as superoxide dismutase, catalase and glutathione

reductase and exogenous antioxidants obtained from diet such as β-carotene, α-tocopherol and ascorbic acid or synthetic such as N-acetyl cysteine (NAC).

NAC is a precursor of Glutathione (GSH), is widely used and has attracted great interest as a thiol-containing antioxidant and modulator of the intracellular redox state (Samuni *et al.*, 2013). In addition, it has been demonstrated that repletion of GSH levels through NAC protects against oxidative stress-induced cell death though scavenging of free radicals (Mayer & Noble, 1994).

1.1.1 Biomaterials and oxidative stress

Over the last few decades, many studies (Mouthuy *et al.*, 2016; Dunnill *et al.*, 2017; Gouzos *et al.*, 2020) have identified significant connections among oxidative stress, inflammation and healing following implantation of biomaterial. Increasing evidence indicates that the production of oxidants and the cellular response to oxidative stress are intricately connected to the fate of implanted biomaterials. The oxidative stress response is modulated by the properties of the biomaterial itself, including its composition, surface characteristics, and degradation byproducts. As both cells and biomaterials can generate and respond to ROS, oxidative stress may represent a key mechanism in the communication between implanted materials and host cells, significantly impacting the biocompatibility of the biomaterial.

Polycaprolactone (PCL) has been widely selected as a biomaterial for bone regeneration due to its biocompatibility, mechanical properties, and slow degradation rate. Unlike other polymers, PCL exhibits excellent processability, allowing for electrospinning and 3D printing, which facilitates scaffold fabrication with tunable porosity and mechanical strength (Woodruff & Hutmacher, 2010). However, its hydrophobic nature can limit cell adhesion, necessitating modifications such as surface functionalization or antioxidant incorporation. Vitamin C, when incorporated into PCL,

not only enhances its bioactivity but also counteracts oxidative stress-induced damage in bone regeneration applications (Oreffo *et al.*, 2016).

To counteract oxidative stress, Vitamin C (ascorbic acid) is a potent antioxidant known to modulate oxidative stress by scavenging free radicals and preventing ROS-induced cellular damage. Beyond its antioxidative function, Vitamin C plays a crucial role in osteogenic differentiation by promoting collagen synthesis, upregulating osteoblast-specific genes such as *Runx2* and *ALP*, and enhancing the deposition of mineralized extracellular matrix (Choi *et al.*, 2019). Studies have demonstrated that the local delivery of Vitamin C within biomaterial scaffolds can enhance osteogenesis while mitigating inflammation, making it a promising candidate for incorporation into bone-regenerative biomaterials (Bose *et al.*, 2019).

Despite these advancements, current strategies for mitigating ROS-related tissue damage include systemic antioxidant administration, surface modifications of biomaterials, and the use of exogenous enzymatic antioxidants. However, systemic antioxidants often suffer from low bioavailability and rapid metabolism, limiting their efficacy at the implantation site (Samuni et al., 2013). While biomaterial surface modifications, such as coating with antioxidant molecules, have shown promise, their stability and long-term efficacy remain a challenge. Encapsulation of antioxidants within biomaterials, as demonstrated with PCL-Vit C, offers a promising solution to sustained ROS regulation, ensuring localized antioxidant delivery without disrupting physiological redox homeostasis (Mayer & Noble, 1994).

Through the integration of these biomaterials and antioxidants, a novel strategy for controlling ROS in bone regeneration can be developed. By incorporating Vitamin C into PCL scaffolds, oxidative stress can be modulated, enhancing osteogenic and angiogenic potential while preserving redox homeostasis.

1.1.2 Co-culture of osteoblast and osteoclast *in-vitro*

The use of co-culture models, in contrast to monoculture, offers the significant advantage of better simulating *in-vivo* conditions, making it a valuable approach in research. However, co-culture systems come with challenges, primarily associated with selecting optimal parameters to support the co-existence of different cell types. Factors such as cell ratio, shared medium, time points, imaging, cellular functions and instruments must be carefully controlled. Additionally, the tools required to distinguish the individual contributions of different cell types present another layer of complexity. Despite these challenges, co-culture systems offer a powerful method to explore cell-cell communication through direct physical contact and the exchange of soluble molecules.

The establishment of an OBs and OCs co-culture system is particularly valuable for investigating the intricate crosstalk between these bone cells, specifically in the context of bone remodelling. This co-culture system allows for an in-depth study of the RANKL/OPG signalling pathway, which plays a critical role in regulating osteoclastogenesis. In this pathway, OBs express RANKL, which binds to the receptor RANK on OCs, promoting OC differentiation and activation. OPG, a decoy receptor produced by OBs, acts as a natural inhibitor of RANKL, thereby regulating osteoclast activity and maintaining the balance between bone formation and resorption.

ROS also mediate critical signaling pathways involved in cellular differentiation and regeneration. The mitogen-activated protein kinase (MAPK) pathway is a key signaling cascade involved in cellular responses to oxidative stress, inflammation, and differentiation. In bone regeneration, ROS have been shown to activate the MAPK pathway, leading to the phosphorylation of ERK, JNK, and P38, which regulate osteoblast proliferation and differentiation (Rodríguez-Carballo *et al.*, 2016). Moreover,

the MAPK pathway mediates the crosstalk between osteoblasts and osteoclasts, playing a crucial role in the balance between bone resorption and formation (Kim *et al.*, 2020). Understanding how Vitamin C modulates this pathway can provide insights into its potential to regulate oxidative stress while enhancing bone healing.

ROS further influence this pathway by modulating the expression of both RANKL and OPG. Elevated ROS levels, often be associated with oxidative stress, increase RANKL expression and reduce OPG production, thereby tipping the balance toward osteoclast activation and bone resorption. Co-culture models of OBs and OCs provide an ideal system to study how ROS impacts the RANKL/OPG pathway, enabling a closer examination of how oxidative stress affects bone cell communication and the bone remodelling process.

1.2 Problem Statement

Bone regeneration continues to represent a significant challenge within biomaterial science, predominantly due to the persistent oxidative stress induced by ROS generated following biomaterial implantation. Excessive ROS generation can lead to chronic inflammation, negatively impacting the integration between the biomaterial and host tissue and consequently impairing effective bone regeneration. Several methods were introduced to mitigate ROS such as selecting healthy patients, ensuring proper biomaterials properties, eliminating inflammation at site of surgery, refining the surgical techniques, yet chronic inflammation triggered by oxidative stress remains a critical barrier.

Current therapeutic strategies, including the systemic administration of antioxidants have been partially successful. However, these approaches face significant

limitations. Systemic antioxidants often suffer from low bioavailability, rapid metabolism, and clearance, reducing their effectiveness at the site of implantation. Moreover, the precise mechanisms through which ROS modulate inflammatory and regenerative pathways following biomaterial implantation are still not fully elucidated, limiting the targeted development of therapies.

Furthermore, existing biomaterials, including widely utilized materials such as polycaprolactone (PCL), have proven its efficiency in tissue engineering but it has limitations due to its hydrophobicity and low bioactivity. The direct local incorporation of antioxidants, such as Vitamin C, into biomaterials remains relatively underexplored, particularly in terms of optimizing antioxidant release kinetics and achieving sustained control over oxidative stress without disrupting necessary physiological processes crucial for tissue regeneration.

Therefore, there is a compelling need for innovative biomaterial-based approaches that effectively integrate antioxidants, particularly Vitamin C, to precisely regulate ROS levels, have high bioavailability and enhance osteogenic differentiation as Vit C supports collagen synthesis, and improve angiogenic processes essential for successful bone regeneration *in-vitro*. Addressing these current limitations will provide significant advancement in the design of smart biomaterials capable of improving the therapeutic outcomes for bone tissue engineering applications.

1.3 Justification of the study

The mechanisms by which oxidative stress is induced following the implantation of biomaterials in bone are still not well understood. It is important to investigate whether the biomaterials themselves play a role in this process and to assess

the effectiveness of both systemic and local antioxidants in controlling ROS at the site of implantation. Current literature reveals several key gaps. The pathways through which ROS is generated in response to biomaterial implantation remain unclear, and it is necessary to explore whether ROS is derived from the biomaterial, the host's biological response, or a combination of both. In addition, while it is known that ROS is produced during and after surgical procedures, there is limited information on the precise levels of ROS generated at the implantation site, particularly in relation to inflammation and healing outcomes.

Another area that requires further investigation is the role of biomaterials, such as PCL, in potentially increasing oxidative stress by promoting ROS production in peri-implant cells. The extent to which this contributes to tissue response and healing has yet to be fully examined. Furthermore, the effectiveness of different strategies for mitigating ROS, whether through local antioxidant delivery or systemic administration, remains unclear. It is not yet determined which approach is most effective in reducing oxidative stress at the site of implantation.

There is also a need to better understand how antioxidants can be optimally released from biomaterials to ensure efficient ROS scavenging. The design of biomaterials capable of delivering antioxidants in a controlled and sustained manner is still an area in need of further research. Finally, while reducing ROS is a key focus, the potential risks of excessive ROS scavenging must also be considered. Over-scavenging could disrupt normal physiological processes and impair tissue healing, yet this aspect has not been adequately studied. Addressing these gaps in knowledge will advance the development of biomaterials that not only enhance bone regeneration but also regulate oxidative stress more effectively, ultimately improving clinical outcomes in bone repair and regeneration.

1.3.1 Research Question

Can vitamin C incorporated polycaprolactone influence oxidative stress related responses in bone regeneration *in-vitro*?'

1.3.2 Null hypothesis

Vitamin C incorporated PCL does not influence oxidative stress cellular responses for mineralization of bone *in-vitro*.

1.4 Objectives of the study

1.4.1 General Objective

To investigate the influence of vitamin C incorporated polycaprolactone toward oxidative stress related responses in bone regeneration *in-vitro*.

1.4.2 Specific Objectives

1. To develop and characterize PCL and PCL-Vit C membranes for bone regeneration.
2. To investigate the influence of ROS in modulating cellular functions in hFOB 1.19 cells on PCL and PCL-Vit C membranes *in-vitro*.
3. To evaluate the effectiveness of PCL and PCL-Vit C membranes as a scaffold for osteoblast differentiation and mineralization *in-vitro*.
4. To investigate the impact of ROS generation on osteogenic marker expressions on PCL and PCL-Vit C membranes.
5. To investigate the MAPK signalling cascades regulating ROS mediated crosstalk between osteoblasts and osteoclasts on PCL and PCL-Vit C membranes *in-vitro* co-culture.

6. To determine the influence of ROS on the regulation of angiogenic-osteogenic coupling in osteoblast-endothelial cells *in-vitro* co-culture.
7. To investigate the MAPK signalling cascades regulating ROS mediated crosstalk between osteoblast and endothelial cells on PCL and PCL-Vit C membranes *in-vitro* co-culture.

CHAPTER 2

LITERATURE REVIEW

2.1 Tissue injury and inflammatory response

2.1.1 Tissue injury in biomaterials implantation

Tissue injury during biomaterial implantation arises from several factors, including surgical procedures, mechanical forces, thermal effects, and the introduction of foreign materials such as sutures and implants. The body's foreign body response (FBR) that occur following the implantation of biomaterials involves blood-material interactions, acute and chronic inflammation, formation of foreign body giant cells (FBGCs) and fibrous capsule formation. These responses are characterized by the infiltration of inflammatory cells and the subsequent release of ROS that can cause pain, reduce the longevity, and compromise the functionality of the implanted device (Anderson *et al.*, 2008). Over time, chronic inflammation develops, fuelled by ongoing oxidative stress due to persistent ROS generation. This oxidative stress is driven both by the ROS production from inflammatory cells and the degradation products of the implanted biomaterials (Tsaryk *et al.*, 2013). The inflammatory response to biomaterials is influenced by their physico-chemical properties, including size, surface charge, shape, and chemical composition, which affect their interactions with tissues and blood (Moghimi *et al.*, 2010; Owens & Peppas, 2006).

2.1.2 The surgical wound and the resulting oxidative stress

Two factors define the redox state or reactivity of the site of implantation before any contact with the biomaterial itself: the degree of pre-existing inflammation in the host tissue and the immediate stress resulting from the surgical wound.

Prior to biomaterial implantation, the surgical procedure and injury create a wound and tissue damage. This cellular and tissue damage results in the release of the intra- and extracellular components in the wound environment, which contribute to increasing the levels of oxidative stress. This partially results from the direct release of existing ROS from damaged cells. At the wound margin, H_2O_2 promotes the killing of invading bacteria and play a role in the rapid recruitment of phagocytic leukocytes from distant sites (Niethammer *et al.*, 2009). Temporary hypoxia may be caused after injury, as restriction in blood supply to tissues can cause a shortage in oxygen. During hypoxia, lack of oxygen triggers a series of metabolic events leading to increased ROS, reactive nitrogen species (RNS) and lipid peroxidation (LPO) production (Fig 2.1).

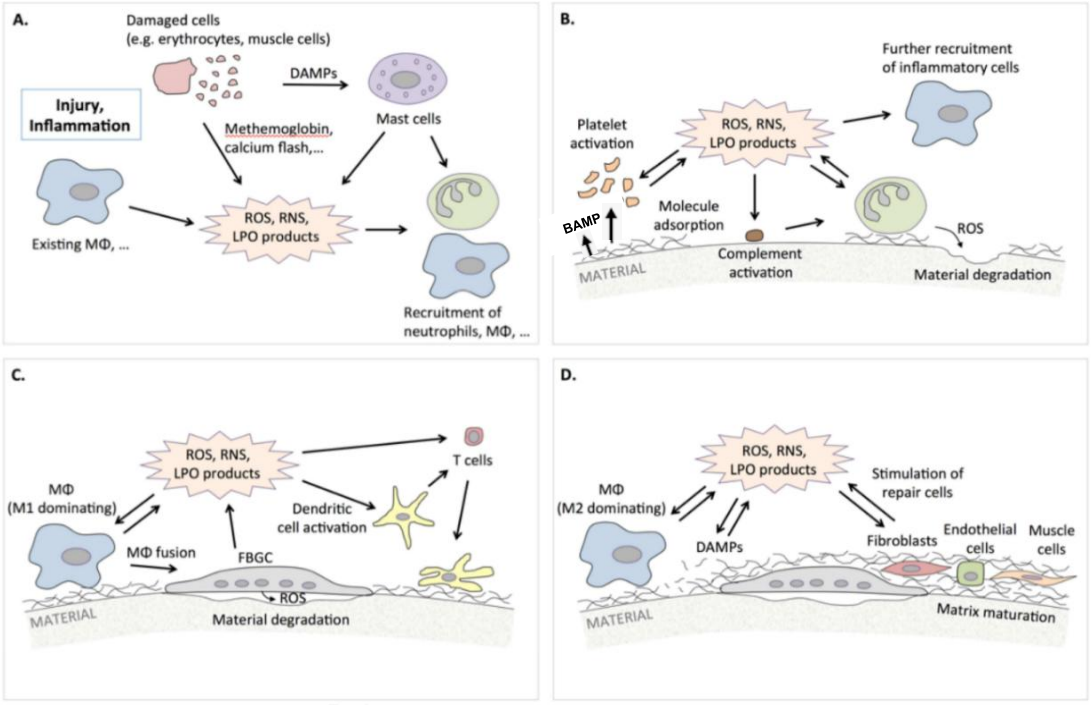


Figure 2.1 Oxidative stress involved during the inflammation and healing phase in presence of a biomaterial. A. prior material-tissue contacts, B. directly following material implantation, C. during the acute and chronic inflammation, D. during healing and tissue remodelling; adapted from Mouthuy *et al.* (2016). BAMP=Biomaterial-Associated Molecular Pattern; DAMPs=Damage-Associated Molecular Patterns; LPO=Lipid peroxidation; RNS=Reactive Nitrogen Species; ROS=Reactive Oxygen Species.

2.1.3 Pre-existing oxidative stress

Tissues affected by trauma or disease often exhibit a baseline level of inflammation before biomaterial implantation, as the body initiates its own healing processes. This pre-existing inflammation likely contributes to oxidative stress. During the healing process, H_2O_2 can activate NF-kB, which in turn promotes the expression of inflammatory genes and NOX enzymes (Gough & Cotter, 2011; Nisimoto *et al.*, 2014). The inflammatory cytokines produced, such as IL6, IL1β, and IFNγ, further enhance inflammatory gene expression and stimulate NOX enzymes via modifications in the NF-kB and protein kinase C (PKC) pathways, leading to increased ROS production (Li *et al.*, 2015). Pathological conditions associated with oxidative stress frequently exhibit elevated oxidant levels. Frijhoff *et al.* (2015) reviewed the oxidative stress biomarkers to study different diseases such as cardiovascular diseases which are marked by heightened inflammation and lipid peroxidation, particularly within lipoproteins, generates various oxidative stress markers. Pre-existing oxidative stress could significantly influence the behaviour of biomaterials, particularly those that are degradable, as they might degrade more rapidly in environments with higher oxidant concentrations. Oliva *et al.* (2015), assessed dendrimer/dextran material-tissue interactions in inflammatory colitis and colon cancer found that the dendrimer/dextran biomaterial compatibility impacted the surface chemistry of surrounding tissues and the biological microenvironment, and this was related to the extent and nature of immune cells in the diseased environment present before material implantation. Therefore, the selection of biomaterials should be based on a comprehensive understanding of the nature and extent of inflammation in the diseased or injured area, due to the high negative impact of oxidative stress.

2.2 Sources and impact of oxidative stress following biomaterial implantation

2.2.1 Sources of ROS production

ROS is generated primarily from two key sources. One source involves the by-products of oxidative metabolism, particularly through mitochondrial respiration. Alternatively, reactive species can also be produced as part of the cellular response to xenobiotics or cytokines, which are released during the body's defense mechanisms (Finkel, 2011).

2.2.1(a) Endogenous ROS sources

Mitochondria is responsible for producing approximately 90% of the body's ATP through oxidative phosphorylation, a process that also makes them a major source of ROS (Li *et al.,* 2013). The generation of ROS primarily occurs within the mitochondria's inner membrane, specifically at complexes I and III of the electron transport chain. Here, ($O_2^{\bullet-}$) are produced as a byproduct of the monoelectronic reduction of oxygen during the oxidation of nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide (FADH2) (Han *et al.*, 2001; Muller, 2000).

During aerobic respiration, mitochondria are a significant endogenous source of ROS, as the electron transport chain leaks electrons that prematurely reduce oxygen to form superoxide radicals instead of water (Li *et al.,* 2013). While these radicals are typically managed by a series of enzymatic reactions within metabolic pathways, they can still contribute to oxidative stress if not adequately neutralized (Han *et al.*, 2001; Muller, 2000).

Another source of ROS production is the electron transfer reactions catalyzed by the mitochondrial P450 systems in steroidogenic tissues (Hanukoglu *et al.*, 1993). These P450 systems are dependent on the transfer of electrons from NADPH to P450. During this process, some electrons "leak" and react with O_2 producing superoxide. To

cope with this natural source of ROS, the steroidogenic tissues, ovary and testis, have a large concentration of antioxidants such as vitamin C (ascorbate) and β-carotene and anti-oxidant enzymes (Hanukoglu, 2006).

When too much damage is present in mitochondria, a cell undergoes apoptosis or programmed cell death (Curtin *et al.*, 2002). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) are involved in ROS generation; there are seven subtypes, NOX1–NOX5 and the dual oxidases (DUOX1 and DUOX2). These transmembrane proteins have in common conserved structural properties that like the carboxyl (–COOH)-terminal site, NADPH and flavin adenine dinucleotide (FAD) binding sites, six transmembrane domains, four heme-binding histidine and amine (–NH2)-terminal transmembrane domains (Bedard & Krause, 2007). ROS modified proteins composed of cysteine, methionine and selenocysteine. One such enzyme that is involved in MAPK functioning is protein tyrosine phosphatase (PTP), which is the target of ROS activity; hence, ROS are indirectly involved in MAPK activation (Son *et al.*, 2011). Upon ROS activation, signalling molecules such as G protein-coupled receptors (GPCRs) and platelet-derived growth factor receptors (PDGFRs) activate enzymes that build NADPH oxidase and when ROS levels rise, they trigger the production of Apurinic/apyrimidinic (AP) endonuclease (APE1), also known as Redox effector factor 1 (APE1/Ref1) a protein complex that activates several signalling pathways. These pathways include p53 for programmed cell death, MAPK pathways for cell growth and stress response, Nrf2 for antioxidant defense, and NF-κB for inflammation. APE1/Ref1 also keeps important transcription factors like AP1, NF-κB, and CREB active by maintaining their reduced state. In essence, ROS act as cellular messengers, helping regulate inflammation, cell growth, and even cell death at balanced levels. However, excessive ROS can lead to damage and disease, which is why

antioxidant defense systems are crucial for maintaining cellular health (Bhattacharyya *et al.*, 2014) (Fig 2.2).

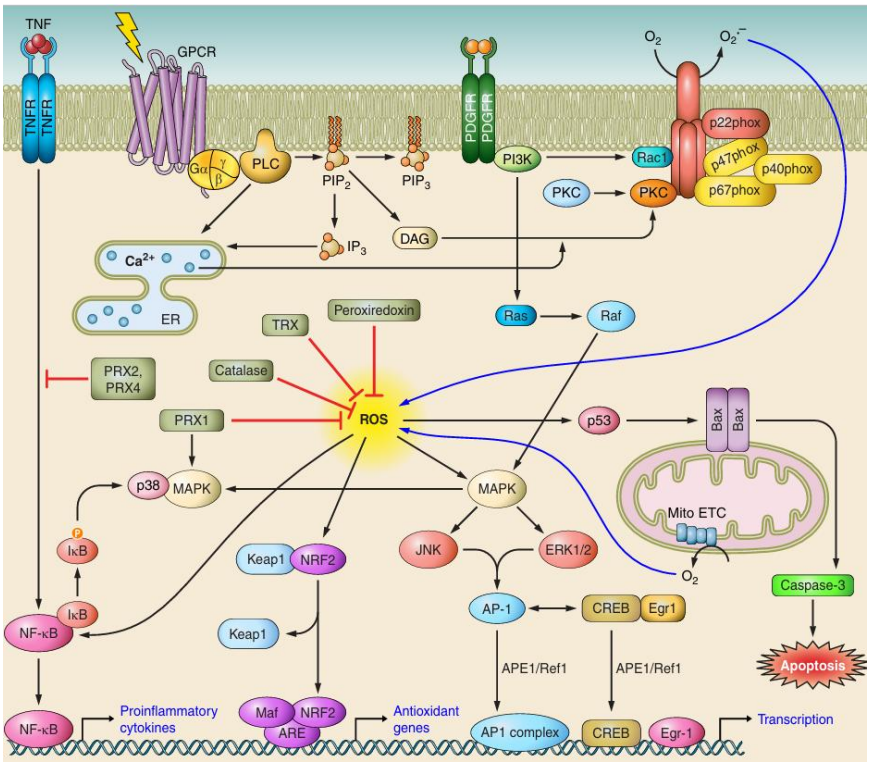


Figure 2.2 Schematic depiction of multiple signalling pathways that generate ROS and the intracellular events activated by ROS accumulation; adapted from Bhattacharyya *et al.* (2014). APE1/Ref1=Apurinic/apyrimidinic (AP) Endonuclease; ARE=Antioxidant Response Element; cAMP=Cyclic Adenosine Monophosphate; CREB=Response Element-Binding, EGR=Early Growth Response; GPCRs=G Protein-Coupled Receptors; JNK=Jun-N-terminal Kinase; MAPK=Mitogen-Activated Protein Kinase; mito ETC=Mitochondrial Electron Transport Chain; NADPH=Nicotinamide Adenine Dinucleotide Phosphate; NF-κB=Nuclear Factor Kappa B; AP-1= Activator Protein-1; Nrf2=Nuclear factor erythroid 2–Related Factor 2; PDGFRs=Platelet-Derived Growth Factor Receptors; PKC=Protein Kinase C; PLC=Phospholipase C; PRX=Peroxiredoxins; RAC1=Ras-related C3 botulinum toxin substrate; Ref1=Redox Effector Factor 1; ROS=Reactive Oxygen Species; TRX=Thioredoxins.

2.2.1(b) Exogenous ROS sources

The formation of ROS can be stimulated by a variety of agents such as pollutants, heavy metals, tobacco, smoke, drugs, xenobiotics, or radiation (Muthukumar & Nachiappan, 2010). Ionizing radiation can generate damaging intermediates through the interaction with water, a process termed radiolysis. Since water comprises 55–60% of the human body, the probability of radiolysis is quite high

under the presence of ionizing radiation. In the process, water loses an electron and becomes highly reactive. Then through a three-step chain reaction, water is sequentially converted to hydroxyl radical (•OH), H_2O_2, $O_2^{\bullet-}$, and ultimately oxygen (O_2) (Fig 2.3).

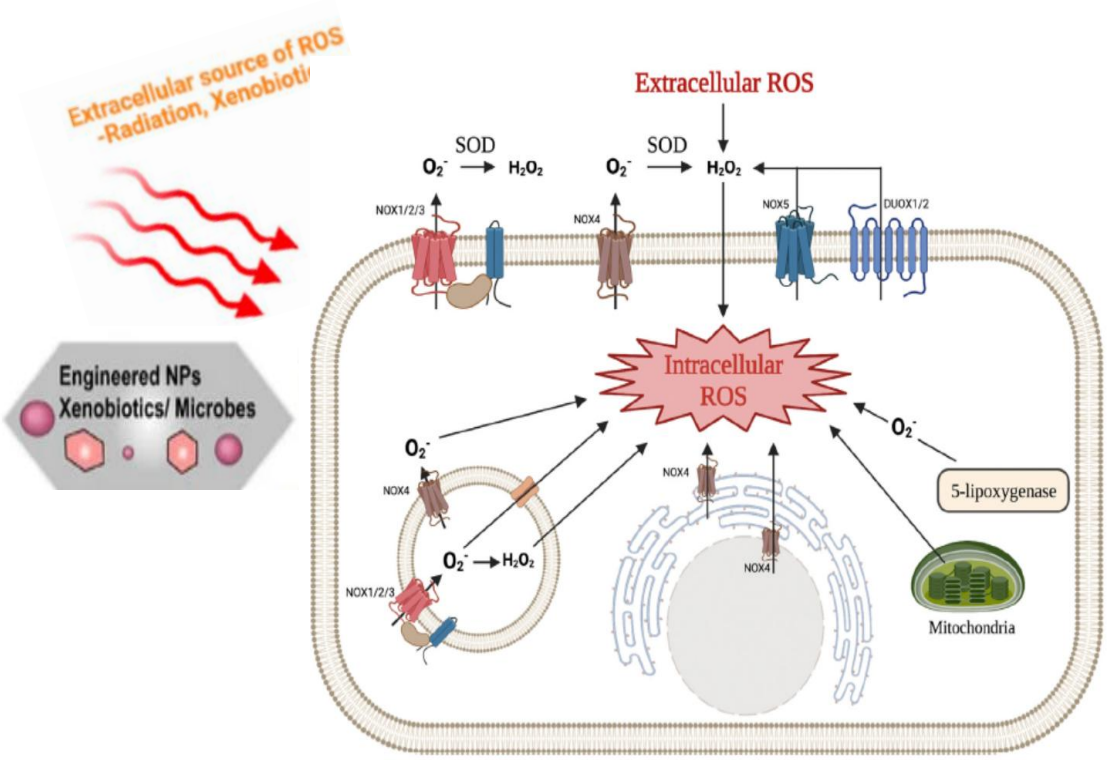


Figure 2.3 Various mechanisms of intra and extracellular ROS production. NOX1, NOX2, NOX3 and NOX4 produce superoxide, which is then converted to hydrogen peroxide in the extracellular space. NOX5 and DUOX1/2 produce hydrogen peroxide directly. Extracellular ROS originated from radiation source, engineered nanoparticles (NPs), xenobiotic and microbes. Adapted and modified from Sheppard *et al.* (2022). DUOX=Dual oxidase enzymes; H_2O_2=Hydrogen peroxide; NOX=Nitrogen oxides; SOD=Superoxide Dismutase.

2.2.2 Approaches for the detection and quantification of ROS

The essential role of ROS in cellular redox balance and their involvement in triggering toxicity and potential cell death (as previously discussed) have prompted the development of advanced methods for their detection and quantification in biological systems. Given the transient nature and limited range of ROS, specialized analytical tools are necessary for accurate measurement (Zhang *et al.*, 2018).

2.2.2(a) Electron spin resonance

Electron spin resonance (ESR) is a key technique that allows for the direct detection of oxygen free radicals. To stabilize these short-lived radicals, specific reagents are employed either through the formation of a covalent bond with the radical, known as a spin trap, or by oxidizing the molecule, termed a spin sensor (Zhang *et al.*, 2018). Spin traps are more frequently used than spin sensors. The most commonly utilized spin trap is 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Finkelstein *et al.*, 1980), which is effective in solution but has limitations in biological systems due to interference from superoxide dismutase (SOD) and ascorbate, reducing the formation of detectable products. Additionally, DMPO's effective concentration range is narrow, requiring ROS concentrations between 20 and 100 mM (Zhao *et al.*, 2005), which limits its sensitivity.

Another spin trap, 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide, can be modified with a triphenylphosphonium group to increase its selectivity for mitochondria (Hardy *et al.*, 2014). However, this spin trap also presents significant challenges, such as low sensitivity (unable to detect ROS concentrations below 50 mM), cytotoxicity, and non-specificity towards superoxide radicals (Abou-Khalil *et al.*, 1985; Kurtoglu & Lampidis, 2009). Moreover, it does not enable ROS quantification and is expensive (Zhang *et al.*, 2018).

2.2.2(b) Chemiluminescent sensors

Chemiluminescent sensors are highly promising tools for ROS, particularly superoxide anions, due to their sensitivity and ease of use (Nandini Yadav & Samir Sharma, 2016). These sensors operate on the principle that when the sensor reacts with ROS, a photon is emitted and detected by a photometer without the need for external

light excitation (Zhang *et al.*, 2018). Lucigenin is one such sensor commonly employed for measuring superoxide anions in macrophages and neutrophils (Vásquez-Vivar *et al.*, 1997). However, lucigenin has limitations, such as its chemiluminescent species being prone to reduction in the presence of flavoprotein reductase, which paradoxically increases superoxide anion levels. Moreover, these chemiluminescent species can react with other molecules, like hydrogen peroxide, thereby reducing the specificity of ROS detection (Zhang *et al.*, 2018).

2.2.2(c) Luminol

Luminol, another chemiluminescent sensor, is less selective because it reacts with hydrogen peroxide, hydroxyl radicals, and peroxynitrite, potentially leading to an increase in these ROS (Faulkner & Fridovich, 1993; Merenyi *et al.*, 1984). This lack of specificity presents a significant challenge for accurate ROS quantification. Superoxide anions can also be detected via cytochrome c (Cc) reduction, which is coupled with spectrophotometry (Dikalov & Harrison, 2014). Superoxide anions reduce Cc from its ferri- to ferro- form, causing a detectable change in absorbance at 550 nm. However, this method's specificity is limited due to the cross-reactivity of superoxide anions with enzymes or reductants, such as xanthine oxidase, ascorbate, and glutathione, complicating accurate quantification.

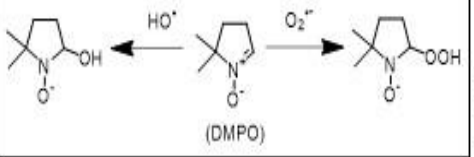
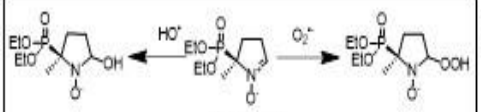
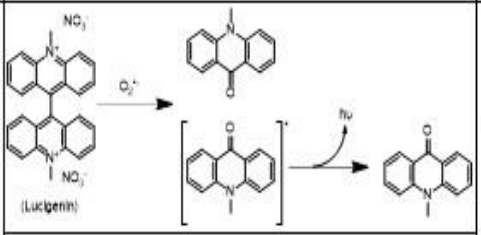
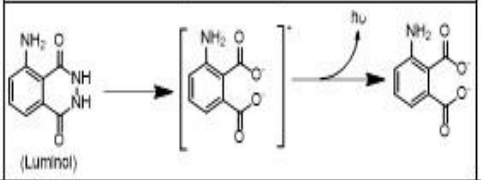
2.2.2(d) Fluorescent sensors

Fluorescent sensors offer an alternative approach for detecting various ROS types. There are two main categories of fluorescent sensors: protein-based sensors and small organic molecules. Protein-based sensors are engineered by combining fluorescent proteins with prokaryotic redox-sensitive proteins (Dikalov & Harrison, 2014). These sensors enable real-time, dynamic detection of redox state changes and

come in different colors, such as green (e.g., redox-sensitive green fluorescent protein types 1 or 2)(Merenyi *et al.*, 1984), yellow (e.g., redox-sensitive yellow fluorescent protein combined with glutaredoxin-1 or modified with three residues)(Hansen *et al.*, 2005), and red (e.g., redox-sensitive red fluorescent protein) (Ermakova *et al.*, 2014). Although biocompatible, these protein-based sensors have limitations, including slow reaction times and low sensitivity, which hinder precise ROS quantification (Zhang *et al.*, 2018).

Organic fluorescent sensors, on the other hand, exhibit altered fluorescent behavior upon reacting with ROS. A wide range of these sensors exists, each with unique properties, including varying emission and excitation wavelengths, selectivity, and the ability to penetrate cells or selectively accumulate in intracellular organelles, such as mitochondria (Gomes *et al.*, 2005). The characteristics of these fluorescent sensors are summarized in Table 2.1.

Table 2.1 Summary of the different methods for ROS analysis and detection; adapted from Adrien (2020).

Method	Reaction	Advantages	Limitations
ESR	 (DMPO)	- Stabilize ROS by addition of covalent bond or by oxidation - Modified DEPMPO can enter selectively in mitochondria with triphenylphosnium group attached on	- Limit of range of ★★ ROS ★ - DEPMPO is more toxic - Need of an external analysis (as NMR) - Non specific
	 (DEPMPO)		
Chemiluminescence	 (Lucigenin)	- Sensitive and easy to operate - Detectable by photometer without excitation by a light source	- Can be reduced by flavoprotein reductase: this reduced form can generate more superoxide anion
	 (Luminol)		- Less selective than Lucigenin - Increases the level of ROS
Cytochrome C	Fe^{3+}-Cc $+O_2^{\bullet -}$ ⟶ Fe^{2+}-Cc $+O_2$	- Form a ferro- form that can be observed using a spectrophotometer - Specific to superoxide anion	- Low specificity due to the cross-reactivity with enzymes or reductants
Fluorescence	Protein-based sensors ⟶ Oxidised protein-based sensor	- Biocompatible	- Slow-reacting - Not very sensitive
	Organic-based sensors ⟶ Oxidised organic-based sensors		

★ DEPMPO =5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide, ★★ ROS=Reactive Oxygen Species.