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**Metal Peroxides: A Promising Paradigm for Advancing Antibacterial Wound
Healing Materials**

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Institut de génie biomédical

Mémoire présenté en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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Ce mémoire intitulé :

Metal Peroxides: A Promising Paradigm for Advancing Antibacterial Wound Healing Materials

présenté par **Dario JOB**

en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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RÉSUMÉ

Le terme "plaie chronique" désigne les lésions cutanées qui ne suivent pas le schéma de cicatrisation attendu, soit en cicatrisant sur de longues périodes, soit en ne cicatrisant pas du tout. Ces complications sont les conséquences de facteurs de risque tels que les infections, l'obésité, la consommation d'alcool et de tabac ou la malnutrition, ainsi que de maladies comme le cancer ou le diabète. Malgré les efforts déployés, les plaies chroniques affectent encore des millions de personnes et coûtent des milliards dans le monde entier. Elles ont des conséquences cliniques désastreuses, notamment la nécrose suivie d'amputations, d'embolies pulmonaires, de thromboses veineuses, de saignements excessifs et de démangeaisons. En outre, les traitements longs, coûteux et lourds, avec des hospitalisations prolongées, peuvent entraîner des angoisses, des dépressions, une fatigue accrue et, plus généralement, une détérioration globale de la qualité de vie des patients.

Grâce aux peroxydes solides, de nombreuses stratégies émergent pour faciliter la cicatrisation des plaies chroniques, telles que les pansements actifs, les hydrogels innovants, les antibiotiques à base de nanomatériaux, etc. Ces matériaux, et plus particulièrement les peroxydes solides à base d'ions métalliques, sont reconnus comme des sources actives d'oxygène en milieu aqueux tout en ayant de puissantes propriétés antibactériennes. Ils sont déjà utilisés depuis plusieurs décennies mais connaissent un regain d'intérêt important. Ils permettent de répondre à des besoins physiologiques qui conduisent à une amélioration de la cicatrisation tout en limitant la croissance de la résistance aux antibiotiques.

Malgré le fort intérêt de la communauté scientifique pour les peroxydes métalliques, les connaissances sont assez pauvres et, pour autant que nous le sachions, il n'existe aucune étude comparant les performances des différents peroxydes métalliques concernant la cicatrisation des plaies ainsi que leurs propriétés antibactériennes. L'objectif de cette thèse est donc d'évaluer les propriétés de trois peroxydes métalliques communs, le peroxyde de calcium (CP), le peroxyde de zinc (ZP) et le peroxyde de magnésium (MP), en mesurant la libération d'oxygène et d'espèces réactives de l'oxygène (ROS), la variation du pH tout en étudiant leurs propriétés antibactériennes et leur cytotoxicité. Le second objectif est de concevoir un pansement fonctionnel pour délivrer de manière appropriée ces peroxydes métalliques à l'aide de films polymères. Les différents résultats confirment le fort intérêt des peroxydes métalliques pour la cicatrisation des plaies et ont fourni une base pour les recherches futures afin de les choisir de manière justifiée et appropriée.

ABSTRACT

The term of "chronic wound" refers to skin lesions that do not follow the expected healing pattern, either by healing over extended periods of time or not healing at all. These complications are the consequences of risk factors like infections, obesity, alcohol and tobacco consumption, or malnutrition and also diseases like cancer or diabetes. Despite ongoing efforts, chronic wounds still affect millions of people and cost billions worldwide. They have disastrous clinical outcomes including necrosis followed by amputations, pulmonary embolisms, venous thromboses, excessive bleeding and itching. Additionally, long, expensive and burdensome treatments with extended hospitalizations can lead to anxieties, depressions, increased fatigue, and more generally to global deterioration of patients' quality of life.

Thanks to solid peroxides, many strategies are emerging to facilitate the healing of chronic wounds, such as active dressings, innovative hydrogels, nanomaterial-based antibiotics, etc. These materials, and more specifically metal ion-based solid peroxides, are recognized as active sources of oxygen in aqueous media while having powerful antibacterial properties. They have already been used for several decades but are experiencing a significant resurgence of interest. They allow physiological needs to be met to lead to an improvement in healing while limiting the growth of antibiotic resistance.

Despite the strong interest of the scientific community towards the metal peroxides, the knowledge is rather poor and, as far as we know, there are no study comparing the performances of the different metal peroxides in wound healing and antibacterial properties. The purpose of this thesis is to assess the properties of three common metal peroxides, calcium peroxide (CP), zinc peroxide (ZP) and magnesium peroxide (MP), by measuring the oxygen and reactive oxygen species (ROS) releases, pH variation while studying their antibacterial properties and cytotoxicity. The second objective is to design a functional dressing to deliver appropriately these metal peroxides using polymeric films. The various results confirm the strong interest of metal peroxides for wound healing and have provided a basis for future research to choose metal peroxides in a reasoned and appropriate manner.

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LIST OF SYMBOLS AND ABBREVIATIONS

AMPs Antimicrobial peptides

AMR Antimicrobial resistance

B.subtilis *Bacillus subtilis*

CP Calcium peroxide

ECM Extracellular matrix

E.Coli *Escherichia coli*

EO Essential oils

HDFs Human Dermal Fibroblasts

hMSC Human Mesenchymal Stem Cells

MP Magnesium peroxide

NPs Nanoparticles

P.Aeruginosa *Pseudomonas Aeruginosa*

PCL Polycaprolactone

PLGA Poly(D,L-lactide-co-glycolide)

ROS Reactive oxygen species

S.Aureus *Staphylococcus Aureus*

SPB Sodium perborate

SPC Sodium percarbonate

WW2 World War two

ZP Zinc peroxide

CHAPTER 1 INTRODUCTION

1.1 Research context

Chronic wounds with high prevalence, ongoing medical care and management, complications and comorbidities are a major public health problem worldwide. These complex medical complications represent a significant challenge for both patients and healthcare professionals. Understanding the underlying mechanisms, preventing their occurrence, and developing effective treatment strategies are therefore essential to improve clinical outcomes and quality of life for those affected. Chronic wounds are skin lesions that do not follow the expected healing process and if persisting require a prolonged period with increased risk of morbidity and mortality. They can result from a variety of causes, including infections, cellulitis, osteomyelitis (bone infection), sepsis (bloodstream infection). Bacterial infections are common complications involving the colonization of pathogenic bacteria which can also form biofilms. To address bacterial infections in chronic wounds, appropriate dressings and antimicrobial therapy are crucial. With the growing concerns of antimicrobial resistance, the dressings should be carefully selected, and the use of antimicrobial agents should be tightly regulated or better avoided, if possible, with alternatives treatments.

1.2 Research objectives

The main goal of this thesis is to develop advanced antibacterial wound healing materials that are free of antibiotics, effective, simple, and inexpensive while promoting wound healing and avoiding complications. Alternative antimicrobial approaches for wound healing have gained attention as potential strategies to combat bacterial infections in chronic wounds. Among them, metal peroxides have gained strong interest as potential antimicrobial agents due to their ability to generate reactive oxygen species (ROS), which exhibit broad-spectrum antimicrobial activity. In addition, their ability to release oxygen is essential for keeping tissue alive after a wound occurs. The objectives are 1) to prepare wound healing materials based on three metal peroxides (calcium peroxide, magnesium peroxide and zinc peroxide) and 2) to compare the antibacterial properties and biocompatibility of the materials.

1.3 Thesis organization

Chapter 2 is a comprehensive literature review including an introduction on the structure and properties of the skin, an overview of the challenges and importance of effective wound healing emphasizing the significance of combating bacterial infections in the healing process, and the different types of wound dressing materials currently used in clinical practice, their limitations, and the need for novel approaches to enhance antibacterial properties more particularly the recent advancements in incorporating metal peroxides into wound healing materials.

Chapter 3 is the purpose and guiding principles of the research and the methodology employed. Chapter 4 is the manuscript of the submitted article, and Chapter 5 concludes the work carried out during the master's degree, as well as potential future work.

CHAPTER 2 LITERATURE REVIEW

2.1 Structure and function of healthy human skin

The skin is composed of three distinct layers: the epidermis, dermis and hypodermis. The outermost layer is the epidermis, which is further divided into five sublayers with unique structures and functions as shown in figure 2.1 [1]. The deepest layer, the stratum basale, contains stem cells that constantly produce keratinocytes [1]. These cells are responsible for producing keratin and regulating calcium absorption. An additional important cell type found in the basal layer are melanocytes, Merkel cells and Langerhans cells. Melanocytes produce melanin, which gives the skin its color and acts as a natural sunscreen [1, 2]. Dendritic cells, such as Langerhans cells, play a significant role in antigen presentation and are part of the mononuclear phagocytic system whereas Merkel cells are mechanoreceptors of light touch and fulfil the sensory function [1-3]. The stratum spinosum consists of a layer of 2 to 10 cells composed mainly of keratinocytes and Langerhans cells [1, 2]. The stratum granulosum is thinner than the stratum spinosum and predominantly consists of granular cells that produce keratohyalin granules and lamellar granules [1]. The main function of this layer is to act as an adhesive and permeability barrier [4]. The stratum lucidum layer turns over only where the skin is thickest, such as the palms of the hands. It is mainly composed of eleidine, produced by keratinocytes, and serves to reduce friction between the stratum granulosum and the stratum corneum [1, 5]. Finally, the stratum corneum is mainly composed of dead keratinocytes and serves as the first protective barrier against the external environment [1, 2].

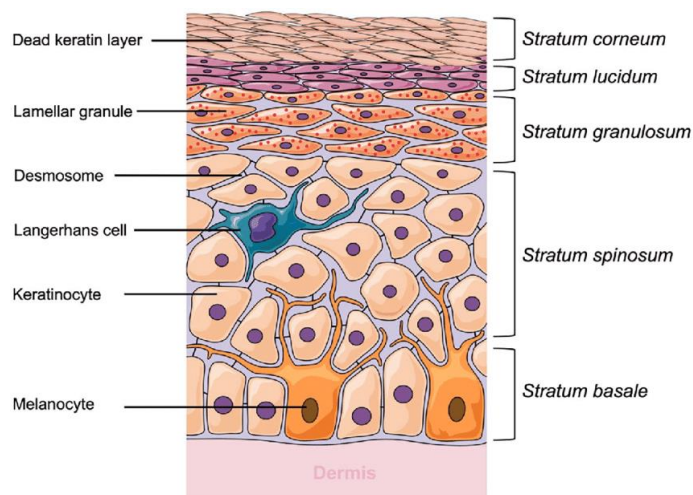


Figure 2.1 Epidermis layer of human skin [6].

The dermis is a layer of connective tissue that is located beneath the epidermis and is attached to it through the basement membrane. It is composed of two layers, the papillary layer and the reticular layer, which merge together without a clear boundary (**figure 2.2**). The papillary layer is the thinner and uppermost layer, made up of loose connective tissue that contacts the epidermis. The reticular layer is thicker, less cellular, and consists of dense connective tissue and bundles of collagen fibers. While the dermis contains various structures such as sweat glands, hair, hair follicles, muscles, sensory neurons, and blood vessels[1]. Additionally, the dermis is the middle layer of the skin and is composed of collagen and elastin, which give the skin excellent mechanical properties such as strength, elasticity, and flexibility [7]. These proteins are produced by fibroblasts, which are the predominant cells in the dermis. The hypodermis lies beneath the dermis and is the deepest layer of the skin. It consists of fat lobules and contains certain skin appendages such as hair follicles, sensory neurons, and blood vessels. This layer is irrigated by blood vessels that provide oxygen and nutrients to the tissues. The hypodermis performs important functions in the body such as thermal insulation, shock absorption and energy storage. [1-3, 8].

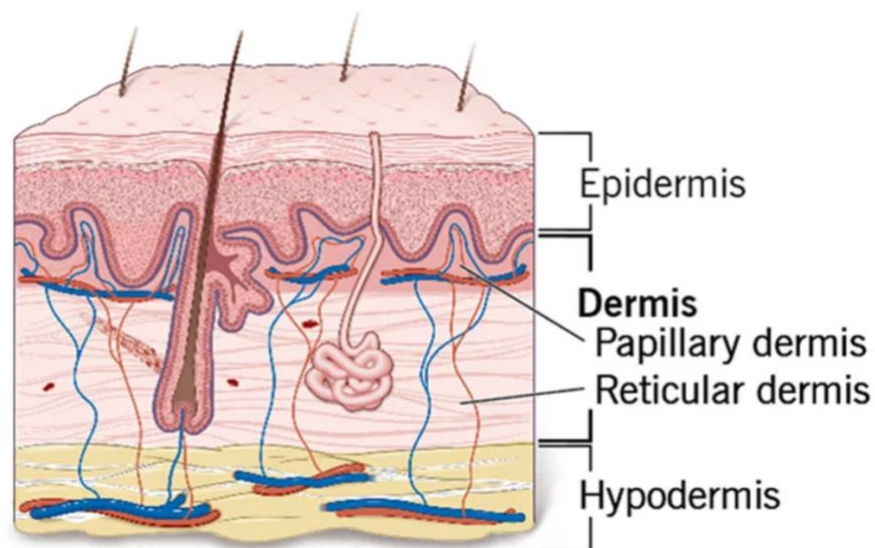


Figure 2.2 Layers of the skin [9].

2.2 The process of wound healing and its phases

While minor wounds tend to heal quickly and easily, acute and chronic wounds can have serious consequences if not properly managed or treated [10-13]. According to the Wounds Canada Institute, the wound care costs at least \$3.9 billion a year in direct costs in Canada. Wound healing process involves complex and highly orchestrated series of cellular and molecular events to restore the function and integrity of damaged tissue. The process can be broadly summarized into hemostasis, inflammation, proliferation, and remodeling [14].

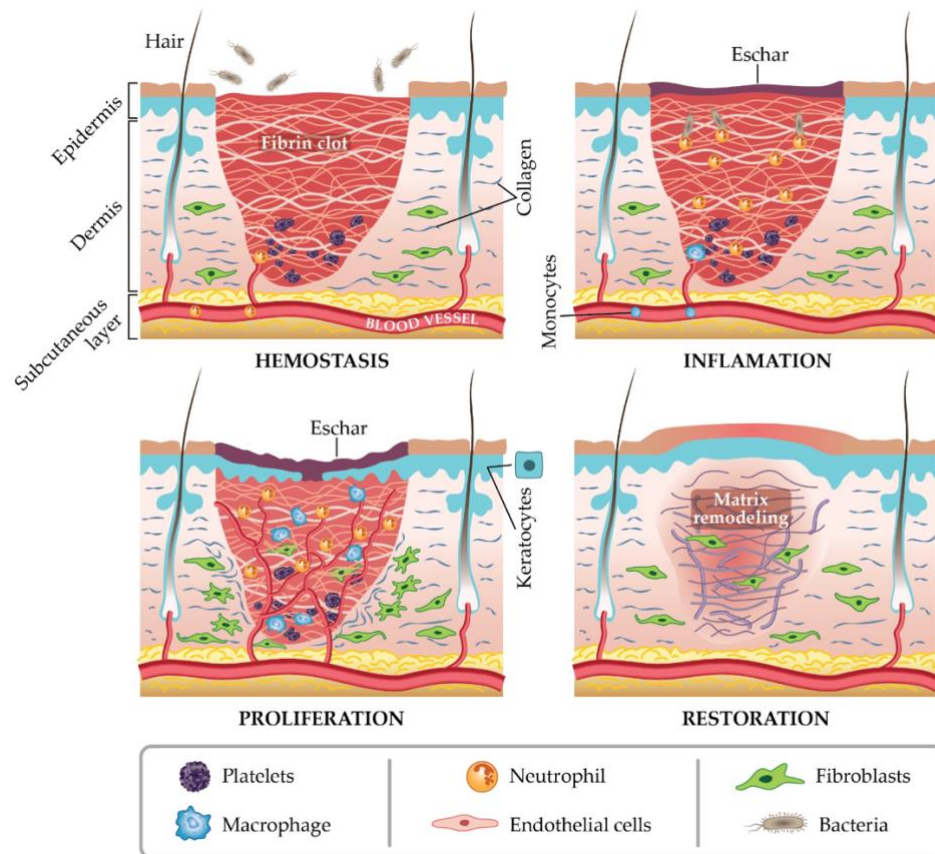


Figure 2.3 Stages of wound healing [15].

Immediately after tissue damage, a cascade of physiological responses occurs. First, hemostasis is the immediate protective mechanism that triggers bleeding to stop. Within seconds, vessels up to 5 mm in diameter become occluded, and within minutes, blood flow is reduced causing tissue hypoxia and acidosis [16]. This process is itself broken down into several steps that allow the blood to move from a liquid state to a thrombus, also known as a blood clot. This mechanism is a complex overlay of procoagulant and anticoagulant processes [17]. As the wound healing process progresses, the clot gradually remodels and disintegrates, allowing the restoration of normal tissue at the site of injury [18]. This first step, in addition to stopping blood flow, establishes a protective barrier to maintain the integrity of the skin. The cells can then migrate to the site of injury and stimulate fibroblast proliferation [19]. The proliferation of fibroblasts takes place during the second phase of wound healing, inflammation. Indeed, after the platelets have allowed the formation of the thrombus during hemostasis, neutrophils accumulate and remove debris and potential bacteria to prevent infection [14]. In addition, neutrophils produce toxic substances such as lactoferrin,

proteases, neutrophilic elastase, and ROS [20], to help destroying bacteria and dead tissue, but can damage cells. Macrophages also play a crucial role in the inflammatory response by recruiting chemoattractant such as chemokines, removing apoptotic cells such as neutrophils, and facilitating the transition to the third phase of wound healing [21, 22]. Although other cells, such as keratinocytes and lymphocytes, are also involved in the inflammatory phase, their functions will not be developed [16]. Once the debris has been cleared during the inflammatory phase, the proliferation phase can begin, usually from 48 hours up to 14 days [23]. There is then an increase in the activation of keratinocytes, fibroblasts, macrophages, and endothelial cells. The migration and proliferation of keratinocytes allows for re-epithelization to cover the surface of the dermis [24]. To reconstruct the epithelium, fibroblasts synthesize and deposit ECM to replace the temporary clot formed during haemostasis [25]. Once enough matrix is deposited, the myofibroblasts, an active form of fibroblasts, produce fibronectin and collagen to contract and close the wound [26]. Localized hypoxia at the wound tissue level (a lack of oxygen supply) generates a whole mechanism of factor expression and release to stimulate endothelial cells to reconstruct new blood vessels (Angiogenesis) [27]. Macrophages then support microvascular endothelial cells in angiogenesis and remodel the new vessel system [28-30]. The last phases of wound healing, the restoration stage, allow the gradual formation of the scar and the reformation of the tissue. The myofibroblasts finish remodeling the ECM and contracting the tissues while the quantities and types of elastin and collagen will vary over time to recover up to 80% of the tissue strength level [14, 31].

2.3 Healing modulating factors

Wound healing is a complex process involving sensitive cells signaling pathways and consequently many risk factors, i.e. systemic factors and local factors can slow down the healing process and lead to chronic wound healing. Local factors have a direct impact on the characteristics of the wound, such as the concentration of ROS or the level of oxygenation whereas systemic factors refer to the general health of the patient.

2.4 Local factors

Oxygen is a key component of wound healing at all stages and for many processes. Low oxygenation is normal and necessary in the early stages of wound healing by inducing hypoxia which is essential for the healing process. Hypoxia allows the activation of ROS and stimulates the

healing process [32, 33]. Oxygenation then helps neutrophils and macrophages to produce ROS and thus contributes to the prevention and reduction of infections. In addition, oxygen also increases the differentiation and migration of keratinocytes as well as their re-epithelialisation, notably thanks to ROS, and increases the production of collagen and endothelial cells, helps with angiogenesis and even collagen synthesis [32, 34]. Prolonged hypoxia (e.g. due to vascular complications, venous insufficiency, arterial disease) results in limited or no oxygen supply and leads to tissue loss, ulcers and gangrene. While oxygen demand is greatly increased during wound healing, cellular processes are slowed or interrupted during hypoxia, collagen cannot be synthesised, ECM cannot be formed, wounds become infected more easily [35]. On the other hand, hyperoxia, a too high level of oxygen, is particularly toxic due to an excessively high production of ROS and can lead to cell death [36]. A common way of defining hypoxia or hyperoxia is, for example, to measure oxygen partial pressure. Generally, hypoxia is considered to be below 60 mmHg and hyperoxia above 120 mmHg. Oxygen consumption during wound healing is very complex to determine, since each cell type has different needs and is present in different quantities at different times. It also depends on factors such as the size of the wound and the severity of the infection. For example, human dermal fibroblasts (HDFs) would consume around 10^{-17} mol/cell/sec of oxygen [37, 38]. There's no precise answer to this, only values on a case-by-case basis. For instance, it has been determined that the consumption per milligram of skin flap for a rat is approximately 0.017 $\mu\text{mol/h}$ [39].

The term ROS refers to species containing two oxygen molecules such as superoxide anions O_2^- , hydrogen peroxide H_2O_2 or hydroxyl radical ($\text{HO}^\cdot$). These species are naturally produced during the wound healing process and play a very important role in the prevention of infections as they have strong antibacterial properties but in too high concentration, ROS kill cells and can lead to necrosis, extend inflammation phase, or even alter angiogenesis [40, 41].

2.5 Systemic factors

Ageing can impair the healing process and is usually associated with a gradual decline in the regenerative capacity of tissues [42, 43]. Chronic diseases such as diabetes also have serious consequences on wound healing and affect many people. In fact, according to the Canadian government, more than 3 million Canadians, or about 9% of the population, have been diagnosed with diabetes, with the prevalence increasing by almost 3% per year [44]. Diabetes causes many

disturbances at all stages of wound healing leading to complications such as disturbances in macrophage activation [45], poor angiogenesis and therefore hypoxia and ulcer problems [46, 47]. This can still affect the individual in many ways such as poor antibacterial defense, disruption of the ECM, damage to DNA and many other consequences that lead to chronic wound healing or worse [48]. Obesity, whatever the cause, is an important risk factor and affects many people since, according to surveys conducted by the Canadian government, more than one in four Canadians suffer from obesity [49]. The reduced mobility of these people as well as the high tension and friction are already factors to be taken into account but beyond its mechanical factors, obesity also affects the healing of wounds by poor vascularization, sensitivity to infection, pressure ulcers and many other consequences [50-52]. Tobacco slows down healing and increases the risk of complications [53-55] as does the consumption of alcohol, which can alter the inflammatory phase, prevent wound closure, angiogenesis and collagen production [56-58]. There are many other risk factors such as medication (anti-inflammatory drugs, steroids, etc.), chemotherapy and radiation exposure. But also, stress, nutrition including protein malnutrition and hormone levels [59-61].

2.6 Nonhealing wounds: Impacts of infections

Although systemic factors are dependent on the individual, bacterial infections are a recurrent risk factor. Infected wounds often involve several types of bacteria working in synergy and worsen situation [62]. Many studies agree that most common pathogens are *Staphylococcus aureus* followed by *Pseudomonas aeruginosa* with a prevalence of 23.3 % and 14,8%, respectively [63, 64]. A significant proliferation of bacteria leads to excessive recruitment and activity of defensive cells [65] leading to abnormal inflammation. The inflammation stage becomes chronic, so the healing process is stopped in the inflammatory phase [63, 65]. In addition to stopping the healing process, the lack of oxygen caused by, for example, too much defensive reaction, leads to the tissue becoming necrotic. While the arterial oxygen pressure (pO₂) is normally in the range of 75-100 mmHg, the pressures achieved in the wound bed are estimated to be 0-20 mmHg compared to 60-70 mmHg in the surrounding tissue [66]. And a minimum pressure of 30-45 mmHg is required to supply oxygen to the bactericidal cells and the generation of these cells could increase up to four times if the pressure reaches levels of 100 mmHg [66, 67]. Thus, bacterial infections can turn healing into chronic wounds with serious consequences such as necrosis.

2.7 Advances in wound dressing: prevent and treat infections

While traditional dressings made of simple cotton strips were not very effective, innovations have made the dressings we are all familiar with more functional. Designs have become more complex with breathable but environmentally protective outer layers to prevent contamination and waterproofing. Absorbent layers have also been added to absorb liquid wound waste such as blood or pus. All these small innovations make the dressings more functional and effective, but they are passive, they protect but do not actively help the wound to heal [68].

With the discovery of penicillin by Scottish scientist Alexander Fleming in 1928, antibiotics are still used today to prevent complications from bacterial infections. There are several antibiotics that can be used depending on the type and severity of the wound, and research has been focusing on incorporating antibiotics into dressings for reducing bacterial colonization, preventing biofilm formation, and promoting a favorable wound environment while offering a more localized delivery directly to the wound.

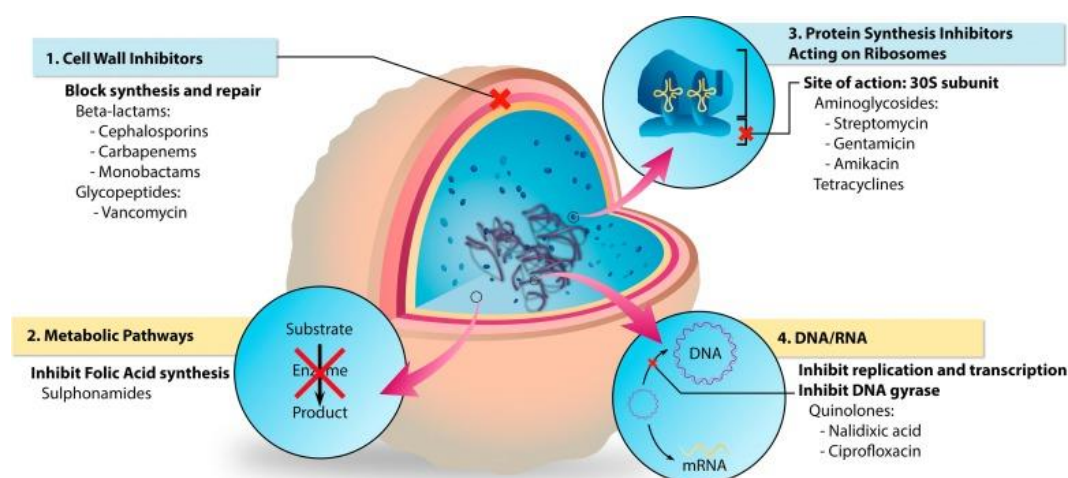


Figure 2.4 Antibiotics in wound dressings [69].

These antibiotics have several strategies to deal with bacteria; Beta-lactams, for example, act directly on the cell wall, while sulphonamides block key metabolic pathways [69] (**Figure 2.4**). Nevertheless, these approaches still contribute to the development of antimicrobial resistance [70]. While researchers develop increasingly sophisticated antibiotics and antibiotic-loaded dressings, the risks associated with antibiotics do not always balance the effective antimicrobial activity with the toxicity. Additionally, bacteria are becoming more resistant to antibiotics, rendering them less

effective [71, 72]. A wide range of alternatives strategies are being developed to provide antibacterial dressings free of antibiotics.

One of these strategies is to use essential oils (EO) such as eucalyptus or rosemary since they have the ability to interact with the cell walls [73], and to make them more effective, they can be nanoencapsulated to act in a controlled and more effective way. Despite these efforts, the use of EO in dressings is not yet optimal, applications must be regular and at very high concentrations [73]. Metal and metal oxide Nanoparticles (NPs) are a promising alternative as antibacterial agents. Their mechanisms of action are not yet fully understood, as they depend on many factors such as their structure (e.g., shape, size) (**Figure 2.5**), but it seems that, generally, metal NPs bind to bacterial cell walls by electrostatic interactions thanks to the negative charges of the metal ions and then release ROS [74-76].



Figure 2.5 Effect of NPs structures on biocompatibility aspects [75].

The production of ROS by NPs can be divided into many reactions, (1) the corrosion of metal NPs, which through secondary reactions like the Fenton reaction or Haber-Weiss will continue to produce ROS (**Figure 2.6**) [77], (2) other mechanisms specific to certain metals such as TiO₂, light

irradiation or via catalytic surface reactions (**Figure 2.6**) [77], and (3) interaction with oxidative organelles or NADPH [75].

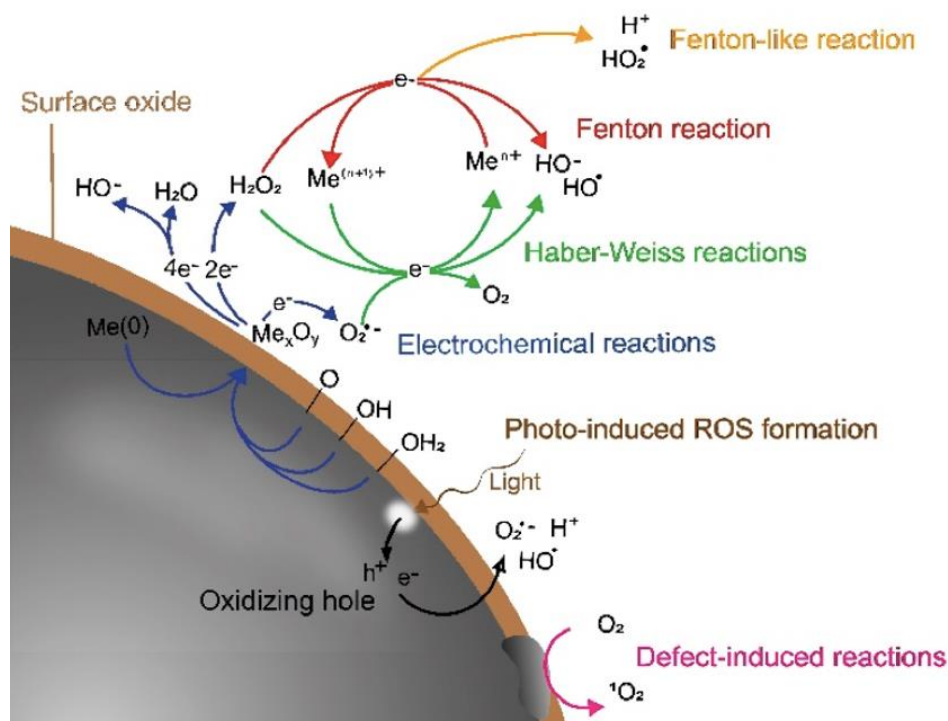


Figure 2.6 The different mechanisms of ROS productions by NPs [77].

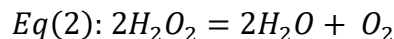
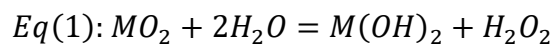
They are generally used in their oxide form such as CuO or ZnO NPs to accentuate the releasing of ROS, but are also more cytotoxic [78, 79]. However, the effects of nanoparticles are highly dependent on bacterial strains. In fact, the different wall structures of gram-positive and gram-negative bacteria, their growth rate and capacity to produce biofilm influence the effectiveness of nanoparticles [76]. All these factors and the lack of understanding on how structure affect bacteria impede the implementation of nanoparticles into an effective universal treatment.

2.8 Metal peroxides

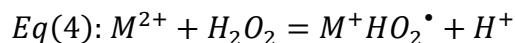
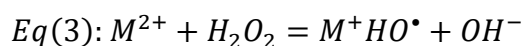
2.8.1 Chemical structures and reactivity

The term peroxide refers to any chemical compound bonded to two oxygen atoms by a single covalent bond. There are many classes of peroxide defined by their chemical structure. There can be organic and inorganic compounds. Metal peroxides are composed with a peroxide ion (O_2^{2-}) bound to metal ions and are generally noted MO_2 . Calcium peroxide (CP) CaO_2 (Ca^{2+} with O_2^{2-}) is

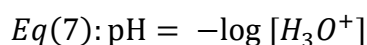
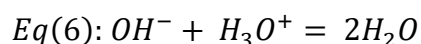
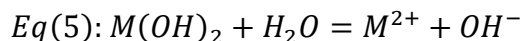
the most known. These compounds are unstable in water and release hydrogen peroxide then oxygen according to equations 1 and 2.



While metal peroxides produce mostly oxygen and hydrogen peroxide, secondary cascade reactions linked to generated hydrogen peroxide can produce additional ROS (such as NPs) with Fenton Like Reaction to produce radical $HO\bullet$ and $HO_2\bullet$ according to equation 3 and 4 [77, 80].



These unique features make metal peroxides a perfect candidate for wound healing by fighting infection while promoting wound reconstruction through the release of oxygen. But, after the release of hydroxide ions (OH^-) by metal hydroxide ($M(OH)_2$) or by Fenton Like Reaction according to equation 1, 3 and 5, the pH of the aqueous solution changes because the concentration of hydroxide ions affects the balance between hydronium ions (H_3O^+) and hydroxide ions (OH^-) according to equation 6 and 7.



2.8.2 Metal peroxide-based materials for wound dressings

Searching library databases quickly led us to understand that the use of peroxides and in particular metal peroxides is a subject that challenges the scientific community for uses in biomedicine with, for example, more than 2,000 articles published in the last 5 years [81]. metal peroxides are used for their oxygen and hydrogen peroxide release properties in many fields of biomedical research such as chemodynamic therapy [82, 83], immunotherapy [84], bones treatments [85] or even various dental treatments such as bleaching [86-88]. Although these applications are varied, one of the most common is the use of metal peroxides as oxygen sources as well as their antibacterial properties for wound healing and other applications related to human tissue.

Table 2.1 Example of metal peroxides -based wound dressings [89-100]

Structures	Peroxides	Studied impacts
Hydrogel	CaO ₂	Oxygen release, fibroblast viability [89]
Sponge	CuO ₂	Antibacterial properties (E. coli, S. aureus, P. aeruginosa) [90]
Hydrogel	CuO ₂	Antibacterial properties (E. coli, B. subtilis) [91]
Cryogel	CaO ₂	Oxygen release, wound closing rate and neovascularization [92]
Polymer film	SPC/ CaO ₂	Oxygen release, collagen deposition, wound closing rate [93]
Hydrogel	CaO ₂	Antibacterial (E. coli, S. aureus), ions release, stem cell behaviors [94]
Polymer film	CaO ₂	Oxygen release, metabolic activity [95]
Polymer film	CaO ₂	Antibacterial (S. aureus), cell viability [96]
Polymer film	CaO ₂	Antibacterial (E. coli), cells viability, oxygen release [97]
Polymer film	SPC	Oxygen release, keratinocyte behavior [98]
Polymer film	SPC	Oxygen release, wound closing rate, fibroblast viability [99]
Spray	CuO ₂	Antibacterial properties (E. coli, S. aureus, P. aeruginosa, MRSA), wound closing rate, collagen deposition, number of inflammatory cells [100]

Research by Suvarnapathaki *et al* has highlighted the potential of metal peroxides -based materials as sources of oxygen to promote wound healing [95]. They designed dressings with CP encapsulated in polyprolactone (PCL), with three different dosages of CP. They quantified oxygen releases over 35 days cultured in tissues culture media. Their samples showed continuous oxygen releases throughout the study, with the two most concentrated samples reaching peak dissolved oxygen concentrations of 24% and 29% on days 19 and 21 respectively. Metabolic activity measurements on myoblast and fibroblast cells carried out by fluorescence measurement with Alamarblue indicated that the most concentrated samples provided the best environment, whereas

controls without CP were deficient under hypoxia. Mollajavadi *et al* prepared alginate-based hydrogels loaded with various concentrations of CP as the active agent [89]. Oxygen release measurements were performed and, during the first 7 days, significant differences in oxygen concentration were observed with peaks in O₂ concentration on the first or second day. At day 10, the differences between the samples and the control were not significant. The two more concentrated samples showed significantly identical results, with higher concentrations than the low CP dosage at each time point, for example around 8 mg/L of oxygen compared with 6mg/L for the low CP dosage and 4mg/L for the control at day 1. They also performed cytotoxicity measurements on fibroblasts on days 1, 3 and 7. The addition of CP to the lowest concentration sample increased viability over time compared with the control without CP and the positive control, while the samples with a high concentration of CP had viability below that of the two controls at each time point. At the end of the study, the viability of the less dosed sample was approximately 180% compared to 50 for the higher CP dosage. Finally, Le Thi *et al* highlighted the antibacterial properties of metal peroxides [94]. Their CP-based hydrogel was effective against gram-positive and gram-negative bacteria. In fact, optical density measurements on *E. coli* or *S. aureus* showed that the addition of CP reduced bacterial activity compared with control. *In vitro* results of a 24-hour wound scratch assay on Human Mesenchymal Stem Cells (hMSC), showed that the addition of CP compared with control significantly improved hMSC migration [94]. Although these measurements were not quantitative, they showed that of the three concentrations of CP, the sample with the highest dosage had the highest migration. The authors suggest that the release of ROS, particularly hydrogen peroxide, from the CP-based samples acts as a chemoattractant between the cells and may help to close wounds more quickly.

CHAPTER 3 CHALLENGES, RESEARCH OBJECTIVES AND GENERAL METHODOLOGY

3.1 Challenges & objectives

The various studies conducted on metal peroxides have allowed the development of various dressings that assist wound healing in different ways depending on the research. This literature review shows the potential of metal peroxides as a promising paradigm for advancing antibacterial wound healing. However, the choice of metal peroxides is quite limited. Early on, CP proved its worth and so researchers continued to use it and today there is a significant amount of research using CP. Although there is indeed some research using other metal peroxides, such as zinc peroxide, the choice of the right metal peroxides for future studies is proving to be quite unclear. While the choice of metal peroxides is proving difficult, the effects observed for the same metal peroxides in different articles are quite different and complicated to compare. For example, distinct oxygen release profiles were observed with a month-long oxygen promotion and a peak around day 20 for PCL-encapsulated CP, and week-long releases for alginate-encapsulated CP. In particular, CP concentration and support material played a key role in the release profile [89, 95]. Furthermore, CP concentration had a different impact on fibroblasts, with a positive influence at higher concentrations for Suvarnapathaki *et al* and a negative one for Mollajavadi's [89, 95].

Despite the fact that the literature is full of interesting examples of the use of metal peroxides for wound healing and the fact that there are a few kinetic studies that can help us choose between two or three metal peroxides [101, 102], none of them give a complete picture of various metal peroxides for medical applications and more specifically for wound healing. Our objective is to manufacture and characterize different films loaded with different metal peroxides: Calcium peroxide (CP), Zinc peroxide (ZP), and Magnesium peroxide (MP). Our hypothesis is that films based on PCL as a hydrophobic barrier will slow and control the solubility of metal peroxides causing different release pattern that will allow to tune the bactericidal effect while ensuring cell survival. We believe that the addition of iron oxide as a catalyst, together with sodium bicarbonate, will help to control the release of hydrogen peroxide. Our final objective is to link the effects of selected metal peroxides on pH, oxygen release and ROS production with bactericidal and cytotoxic effects.

3.2 General methodology

Polymeric films were produced by mixing metal peroxides with sodium bicarbonate and calcium dihydrogen phosphate monohydrate. This powder mixture was incorporated in a chloroform:PCL solution to produce metal peroxides -based polymer films by evaporation. In addition to physico-chemical characterization, preliminary tests were carried out to check the antibacterial effects with measurements of inhibition zones on Agar plates with E.Coli (**Figure 3.1**).

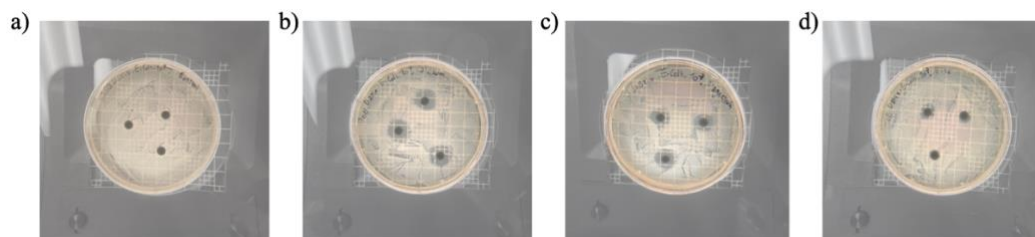


Figure 3.1 Agar plate disk inhibition test for E.Coli: Control (a), CP (b), MP(c), ZP(d).

In addition to inhibition zone diameter, a plate count test using *P. Aeruginosa* and *S. Aureus* were carried out and cytotoxicity tests using human fibroblasts were performed with alamarBlue.

Oxygen was measured with an optical probe, hydrogen peroxide and ROS with chemical probes.

CHAPTER 4 ARTICLE 1: METAL PEROXIDES: A PROMISING PARADIGM FOR ADVANCING ANTIBACTERIAL WOUND HEALING MATERIALS

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Abstract

Wound healing and prevention of bacterial infections are critical aspects of modern medical care. In this study, we engineered wound healing and antibacterial materials by incorporating peroxide dopants into a polymeric film. The utilization of peroxide species offers several advantages, including their inherent antibacterial properties and their potential to facilitate wound healing processes. A known concentration of calcium (CaO₂), or magnesium (MgO₂) or zinc (ZnO₂) peroxide was incorporated under similar conditions in a biocompatible polymeric film using a versatile and cost-effective fabrication method, allowing for optimization of the material's antibacterial efficacy while minimizing cytotoxicity effects. To evaluate the antibacterial efficacy of the peroxide-doped film against common wound pathogens, we conducted bacterial culture experiments. The films efficiently prevents the growth of both Gram-positive (*S. Aureus*) and Gram-negative (*P. Aeruginosa*) bacteria. The films effectively killed over 99% of bacteria. We also examined the material's sustained release of reactive oxygen species and oxygen, and pH variation which contributes to its prolonged antibacterial activity. In addition to antibacterial properties, we performed *in vitro* experiments utilizing human dermal fibroblasts. The films demonstrated a moderate degree of biocompatibility towards fibroblast. Overall, given unique combination of

antibacterial efficacy and biocompatibility, these films represent a promising advancement for various medical applications, including wound dressings, bandages, and other healthcare products. Further studies involving *in vivo* evaluations are warranted to fully explore the translational potential of peroxide-based materials.

4.1 Introduction

Antibiotics developed very rapidly after their discovery nearly a century ago [103], and today there are hundreds of them, roughly classified into penicillins, cephalosporins, aminoglycosides, tetracyclines, macrolides, and fluoroquinolones. However, soon after their introduction, antimicrobial resistance (AMR) was observed, resulting in approximately 5 million deaths from AMR worldwide today [104]. New strategies such as antimicrobial peptides (AMPs) lead to disruption of lipid bilayer structures [105-107]. However, while showing promise, resistance to AMPs has also emerged [108, 109]. Improvements in bactericidal materials that physically destroy bacteria have subsequently attracted renewed interest, especially if they can be applied topically or as implantable coatings. For example, as the development of photography facilitated the study of colloidal silver, there were several reports of its use in the treatment of erysipelas, strep, gonorrhea, and typhoid between the 1900s and 1930s. This approach was largely abandoned when the effectiveness of antibiotics became apparent, so between 1930 and 1980 there were very few reports of nanosilver or colloidal silver for bacterial infections. But in this century alone, there are more than 10,000 publications [110]. In addition to silver nanoparticles, weak antiseptics such as potassium permanganate have been used, but more effective compounds such as carbolic acid, mercuric chloride, and hydrogen peroxide are of limited value due to their corrosive or toxic properties. Sodium hypochlorite and boric acid in a neutralizing solution provided an effective but chemically unstable direct contact antiseptic for wounds. To overcome this instability, a slow drip continuous flush known as the Carrel-Dakin process was developed [111].

Due to the low solubility of zinc oxide and zinc hydroxide, zinc peroxide is one of the least corrosive solid peroxides. It decomposes slowly in aqueous conditions liberating oxygen and hydrogen peroxide and was used extensively during WW2 in the care of wounds or other lesions caused, or secondarily infected, by anaerobic streptococi, tetanus and anaerobes of the gas gangrene group, *B. melaninogenicum*, *B. fusiformis*, *Actinomyces*, and certain *Spirochaetes*. However, its effectiveness is limited by loss of activation, it needs to be warmed before use (presumably to

break down any carbonates) and reapplied daily, and it sticks to the wound and can cause a foreign body reaction. To overcome these problems, Reid and Altemeier [112] developed a formulation with delayed release of oxygen. Shortly after the widespread use of antibiotics, a 1952 review article claimed that zinc peroxide was superior to sulfonamides and antibiotics in the treatment of gastric ulcers; synergistic gangrene, only after excision; decubitus ulcers; other ulcers with high rates of infection; diabetic gangrenes and ulcerative stomatitis. Afterwards, the work shifted to other compounds such as benzoyl peroxide [113].

As the threat of AMR increases, solid peroxides, such as nanoparticulate formulations or metal peroxides, are being reconsidered. In fact, inorganic peroxides are considered to be effective alternative sources of hydrogen peroxide (H_2O_2), which can release H_2O_2 in acidic environments. For example, copper peroxide has been evaluated for wound healing or chemokinetic therapy [90, 114]. Metal peroxides can also release O_2 , which can maintain elevated oxygen levels for several days [94, 102, 115-117]. Controlling the decomposition products of metal peroxides and reducing the rate of oxygen evolution has been the goal of some previous studies. For example, nanoscale metal peroxides are used [114, 115, 118]. Another approach is to encapsulate them in polymers that provide cells or tissues with oxygen for longer periods of time and add catalysts to limit H_2O_2 production [119-122].

The purpose of this study was to provide comparative data of a selection of metal peroxides in a polymeric film system following the previous identification of parameters affecting hydrogen peroxide or oxygen release [123] and the development of a low toxicity formulation with a catalyst to reduce excess hydrogen peroxide formation and a buffer to prevent excessive pH elevation. Calcium peroxide (CaO_2), magnesium peroxide (MgO_2), and zinc peroxide (ZnO_2) were formulated and evaluated for their antimicrobial activity against *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two of the most common bacteria in a variety of microbial infections and they may respond differently to metal peroxides [124]. Since each metal peroxide has different release properties, kinetic measurements of oxygen, H_2O_2 and ROS release and pH variation were conducted and associated to their antibacterial and cytotoxic effects.

4.2 Materials

Zinc peroxide ZnO_2 (ZP) (481424), magnesium peroxide MgO_2 (MP)(433624), polycaprolactone $((\text{C}_6\text{H}_{10}\text{O}_2)_n$ (PCL)(440744), hydrogen peroxide solution H_2O_2 (30%) (1.07298), sodium bicarbonate CHNaO_3 (S5761), iron (II, III) oxide (637106), chloroform ($\geq 99\%$ CH_3Cl_3) were purchased from Sigma-Aldrich. Calcium peroxide CaO_2 (CP) (UN1457) was obtained from FMC Corporation and calcium dihydrogen phosphate monohydrate $\text{Ca}(\text{H}_2\text{PO}_4)$ (AB119788) from abcr GmbH. Phosphate Buffered Saline (PBS) and fluorescein H2DCFDA (D399) were purchased from ThermoFisher Scientific (10010031).

4.3 Methods

4.3.1 Composite Films

To encapsulate the peroxides within PCL films, each component was sieved through a $74\mu\text{m}$ sieve (ASTM 200 mesh, Gilson company) and added in 100ml of PCL solution at 10% w/v (**Table 4.1**).

Table 4.1 Weight percentage (wt%) of the components encapsulated in PCL.

	Peroxide	Fe_3O_4	CHNaO_3	$\text{Ca}(\text{H}_2\text{PO}_4)_2$	Total
Sample	13.7	13.7	3.3	2.7	33.4
Control	0.00	15.9	3.8	3.1	22.8

Samples with calcium, magnesium or zinc peroxides were referred to as CP, MP and ZP films respectively, and the control sample, C film.

The mixture was stirred until homogeneous, poured into a 190×100 mm Pyrex crystallizer (PYREX, No. 3140) and left to dry, covered with aluminium foil, at room temperature in a safety hood for 24h as illustrated in **Figure 4.1**.

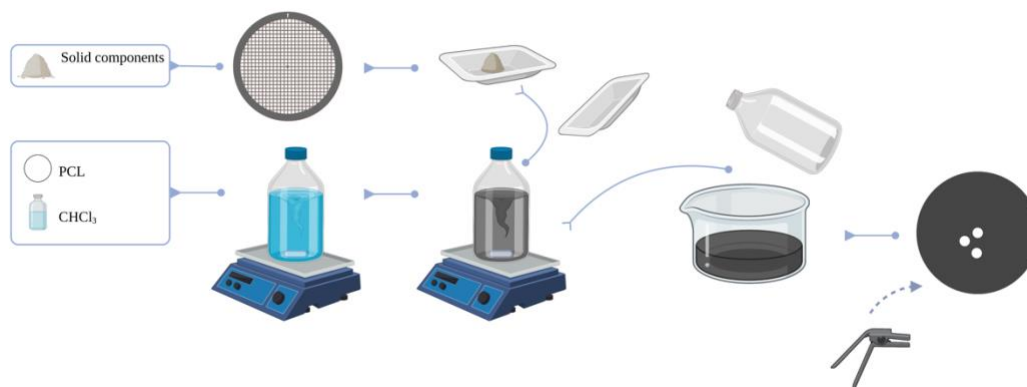


Figure 4.1 Schematic illustration of the composite film processing steps. After sieving the peroxides, all the components are added into the PCL-chloroform solution and mixed thoroughly before being poured into a crystallizer and left to dry. Composite films were generated using a circular hole paper-punch for experimentation (diameter of 6 mm).

4.3.2 Composite characterization

The microstructure of the surfaces of the different composites was examined using scanning electron microscopy (15kV in SE mode, Hitachi-Hightech TM3030 Plus SEM). The porosity was estimated from SEM images with ImageJ software by adjusting the threshold to highlight the pores. Thermogravimetric analysis (TGA, TA instrument, SDT600) was performed under a nitrogen atmosphere using a heating rate of 4.00 °C/min up to 1100°C. The swelling index of the samples was measured by drying 6 mm diameter circular specimens of film (N=3). The samples were dried at 60°C for 4 hours, then the dried samples were weighed and then immersed in milliQ water for 24h. Then, the soaked samples were dabbed dry and reweighed. Swelling index was calculated according to Equation 8:

$$\text{Swelling Index (\%)} = \frac{W_s - W_d}{W_d}, \quad \text{Eq 8}$$

Where W_s is the immersed weight and W_d the initial weight.

4.3.3 Release experiments

To analyze the decomposition behaviour of the different samples, the conversion of the metal peroxides to O_2 and H_2O_2 was evaluated at 1, 2, 3, 4, 24 and 48 hours. Circular films of 6mm were placed in sealed 1.5mL Eppendorf, filled with PBS. Oxygen measurements were made with an oxygen probe (FOSPOR-AL300) connected to the NEOFOX-GT oxygen sensor (Oceaninsight).

The probe was calibrated at two points using nitrogen saturated PBS (0% oxygen) and (20.9% oxygen) PBS. A peroxide assay kit (Pierce™ Quantitative Peroxide Assay Kit, ThermoFisher) was used to measure H₂O₂ concentration. An eight-point standard curve was prepared by diluting 30% H₂O₂ between 0 and 500 μM in PBS to interpret absorbance values. For each sample measurement, 5 μL of the PBS solution in which the sample was inserted into a microplate well, and then 150 μL of reagent solutions were added. The microplate was protected from light by covering it with aluminum foil for 15 minutes at room temperature. Absorbances were measured at 595 nm with a plate reader (ThermoScientific, Multiskan SkyHigh). The pH of the PBS was measured using pH probe (Mettler Toledo and a Sartorius sensor) calibrated at 3 points (4, 6 and 10). Finally, the release of all ROS species was evaluated by immersing the 6 mm circular films into 15 ml vials filled with 4 mL of PBS using a chemically reduced form of fluorescein H₂DCFDA as ROS indicator. A 25 μM reagent solution of H₂DCFDA was produced by dilution with 99.9% ethanol and used as a probe to measure ROS. 200 μL of the sample solution was added into a black microplate (Corning, 3915) and 5 μL of reagent solution was then added and incubated for 10 min wrapped in aluminium foil at room temperature. ROS release was measured at excitation and emission wavelengths of 488 and 525 nm, respectively. Before each measurement, the solutions were vortexed to be homogeneous.

4.3.4 Cytotoxicity assays

Human Dermal Fibroblasts (HDF) cells were provided by the Dr. Joshua Vorstenbosch lab (RI-MUHC, QC Canada). The extraction, expansion, and usage of these HDF cells received Institutional Review Board (IRB) approval by the MUHC Ethics Research Board (approval #MP-37-2020-5995). HDF cells were used between passages 5-8 and subcultured after reaching 75% confluency. HDF cells were grown in DMEM media (Gibco) completed with 5% Fetal Bovine Serum (Gibco) and 1% PenStrep (Gibco). HDF cells were introduced to each sample (n=3) after being plated in a 48-well plate (ThermoFisher) at a cell density of 3×10^5 . To measure the relative cell viability, alamarBlue reagent was used at 1:10 ratio in the completed media and incubated for 1 hour at 24-, 48-, and 72-hour timepoints following plating and introduction to each film condition. The incubated solutions from each well were transferred into a 96-well black UV F-bottom plate with top and bottom fluorescent readings measured at emission/excitation wavelengths of 560/590 nm (ThermoFisher Varioskan Lux).

4.3.5 Plate count assays

S. Aureus (ATCC 6538) and *P. Aeruginosa* (ATCC 15442) (Cedarlane Laboratories, ON, Canada) were stored at -80°C until required. To prepare stock cultures, 5 μL of thawed bacteria was inoculated in 5 mL of LB (Luria-Bertani) broth and incubated at 200 rpm, 37°C for 18 h. The initial concentrations were estimated to be 3×10^{10} and 9×10^9 cfu/mL for *S. Aureus* and *P. Aeruginosa*, respectively. Then the bacteria strain stock cultures were diluted with nutrient broth (NB 1/500) to achieve the final concentrations of 10^5 cfu/mL. NB 1/500 were adopted from ISO 22196:2011 by dissolving 3.0 g of meat extract, 10.0 g of peptone and 5.0 g of sodium chloride in 1 000 mL of distilled or deionized water. The obtained solution was diluted with Milli-Q water to a 500-fold volume and adjust pH between 6.8 to 7.2. The antibacterial efficacy of the resulting samples was examined through the suspension method. In this respect, CP, MP and ZP, and C films were cut as disks with a size diameter of 6 mm and suspended into the 5 ml diluted bacteria species. The samples were incubated at 37°C , 200 rpm and at varied incubation times, 24 h, 48 h and 72 h. Control samples were considered under similar conditions, excluding samples with an antibacterial agent. At the end of the incubation period, 100 μL of aliquots were taken to prepare 4 serial dilutions with a dilution factor of ten in the saline buffer. Then, the viable bacterial counts were enumerated by spreading 100 μL of diluted aliquots onto the agar Petri dishes, incubated overnight at 37°C . All experiments were repeated in triplicate. Viable bacteria concentration was calculated considering all dilutions made according to Equation 9:

$$\text{Concentration (cfu.ml}^{-1}\text{)} = \frac{n \times d}{V}, \quad \text{Eq 9}$$

where n is the number of colonies (average of 3 replicates), d the dilution factor and V the volume of culture plate in mL ($V=100 \mu\text{L}$). A solution without samples was used as a basis for comparison referred to as the blank.

4.3.6 Statistical analysis

All results were expressed as mean $\pm$ standard deviation ($N=3$) and were normalized by molar mass. A p-values $p < 0.05$ were considered statistically significant. Statistical significance was assessed by one-way ANOVA.

4.4 Results

4.4.1 Physicochemical characterization of the composite films

We assembled a library of 3 types of composite films using zinc-, calcium-, and magnesium peroxides and by combining other components to minimize the cytotoxicity of the samples attributed to H_2O_2 and pH. To moderate this, iron oxide, i.e. peroxide catalyst and calcium monophosphate were added to control the reaction rate [123], and pH acidity compensated by adding sodium bicarbonate [125]. Scanning electron micrographs (**Figure 4.2**) indicated the microstructure of the surface of the samples. All the samples were microporous in the range 50-150 μm and consisted of micro particles embedded in the polymer film. Porosities were estimated by image analysis to be around 9%, 5%, 8% and 1 % respectively for C, CP, MP and ZP.

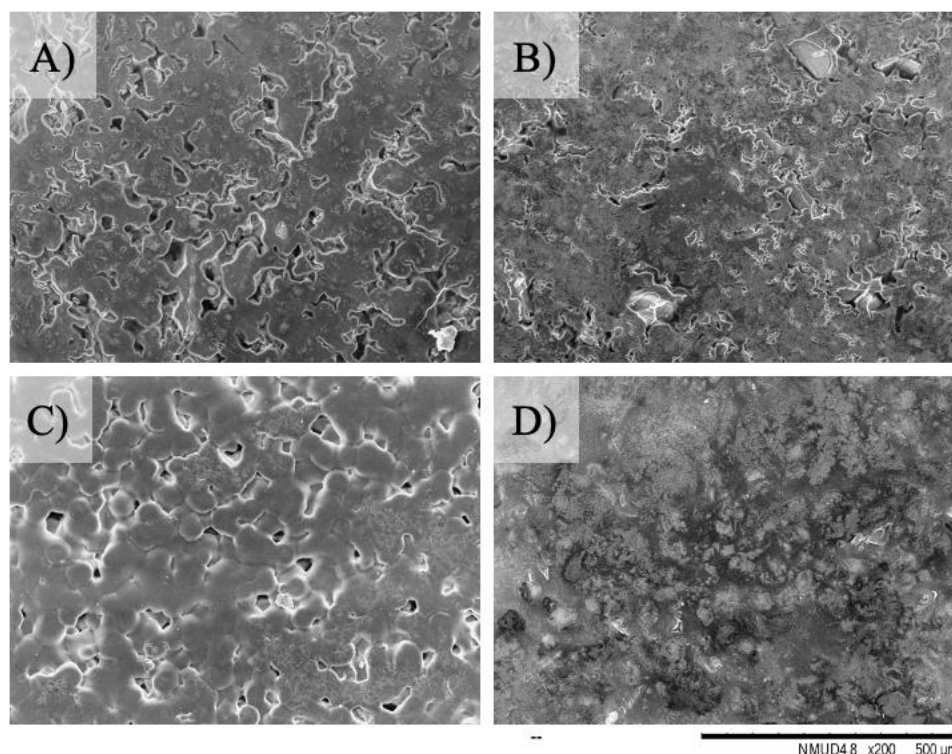


Figure 4.2 Scanning electron micrographs illustrating the microstructure of the control and experimental films: A) Control (C), B) Calcium Peroxide (CP), C) Magnesium Peroxide (MP) and D) Zinc Peroxide (ZP).

The thermal behaviour of the samples were studied using thermogravimetric analysis to confirm the composition of the composite (**Figure 4.3A**). A two-step degradation process for each

materials was observed and attributed to the loss of water and decomposition of NaHCO_3 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ which take place at 100°C and represent a total mass percentage of about 6 to 6.9 according to table 4.1 [126]. Around 300°C , samples start to lose a lot of weight with the degradation of PCL and decomposition of metal peroxides [127, 128]. For CP, there is a shoulder around 350°C which should correspond to the first step of calcium peroxide degradation, then the second shoulder around 500°C is the end of this first degradation and finally the third shoulder would correspond to the beginning of the second step of calcium peroxide degradation. For ZP, no shoulder is observed, PCL and zinc peroxide degradation are confused [128]. For MP, a small shoulder appeared during the degradation of PCL around 350 degrees which corresponds well to the degradation of magnesium peroxide [129]. The swelling index was evaluated for each sample and the results are shown in **Figure 4.3B**. Control lost less than 1% of mass while CP lost about 2%. ZP and MP lose more than 4 and 5% mass respectively.

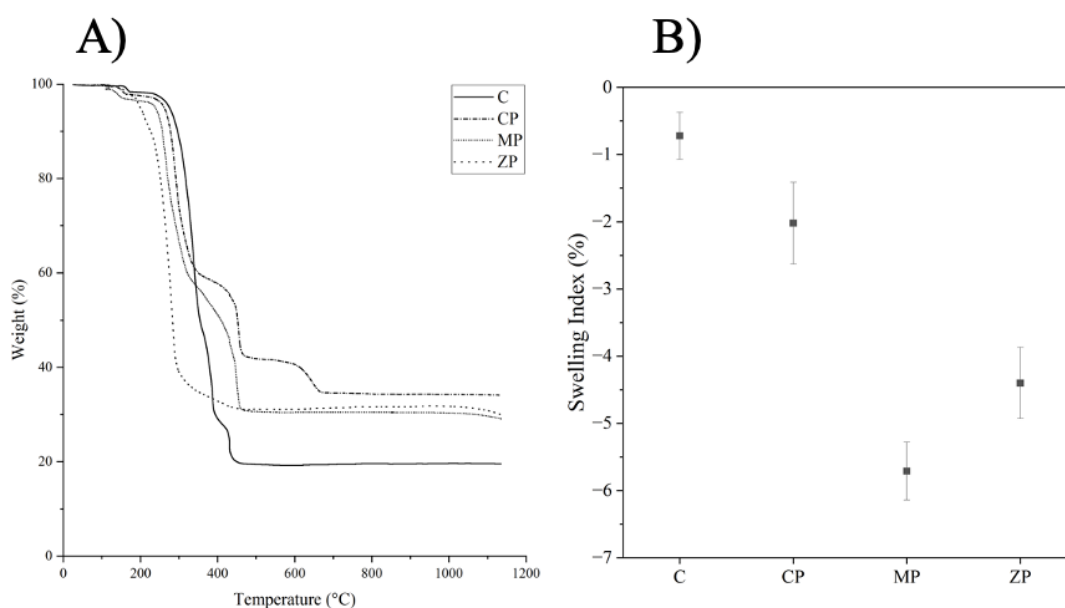


Figure 4.3 A) Thermogravimetric analysis, B) Swelling index after 24 hours.

4.4.2 Product degradation release

The oxygen release from the different films was investigated (**Figure 4.4A**). Unsurprisingly, the results show that the metal peroxides-based samples generate oxygen, whereas no oxygen was measured in media with control film. For each sample, the stronger oxygen release occurs during the first hour and continued to rise gradually until the first and second days. CP produced by far

the largest oxygen released ~3 times more than the other peroxides, with peak concentrations up to about $90 \mu\text{M.mol}^{-1}$ whereas MP and ZP reached maximal values of $\sim 20 \mu\text{M.mol}^{-1}$ and $40 \mu\text{M.mol}^{-1}$, respectively.

The antibacterial properties of metal peroxides are thought to be at least partially due to their release of ROS and more particularly H_2O_2 [130]. The amount of ROS and the initial control values were used as a baseline. The variation in ROS concentration for MP was not statistically significant ($P < 0.05$). For CP and ZP, there was a very slight upward trend and a significant ($P > 0.05$) increase after one day *in vitro*. However, H_2O_2 concentration (**Figure 4.4C**), differed considerably; for CP and MP, the concentration of H_2O_2 gradually increased during the first few hours and then fell at days 1 and 2, whereas for ZP, the concentration increased throughout the experiment. The maximum reached for MP and ZP are about $50 \mu\text{M.mol}^{-1}$ respectively at hour 4 and day 2, while CP had higher concentrations up to about twice as much i.e. $100 \mu\text{M.mol}^{-1}$.

Finally, the pH of the solutions in which the samples were deposited to evaluate their release was also measured since the pH plays an important role in the healing of wounds and especially in the antibacterial effects. The results in **Figure 4.4D** show that ZP has no effect on pH as does the control. For CP and MP, however, the pH changes dramatically after 24 and 48 hours. CP reaches values of about 9.5 and 10 on days 1 and 2, while MP has values of about 8.3 and 8.8 at the same time points.

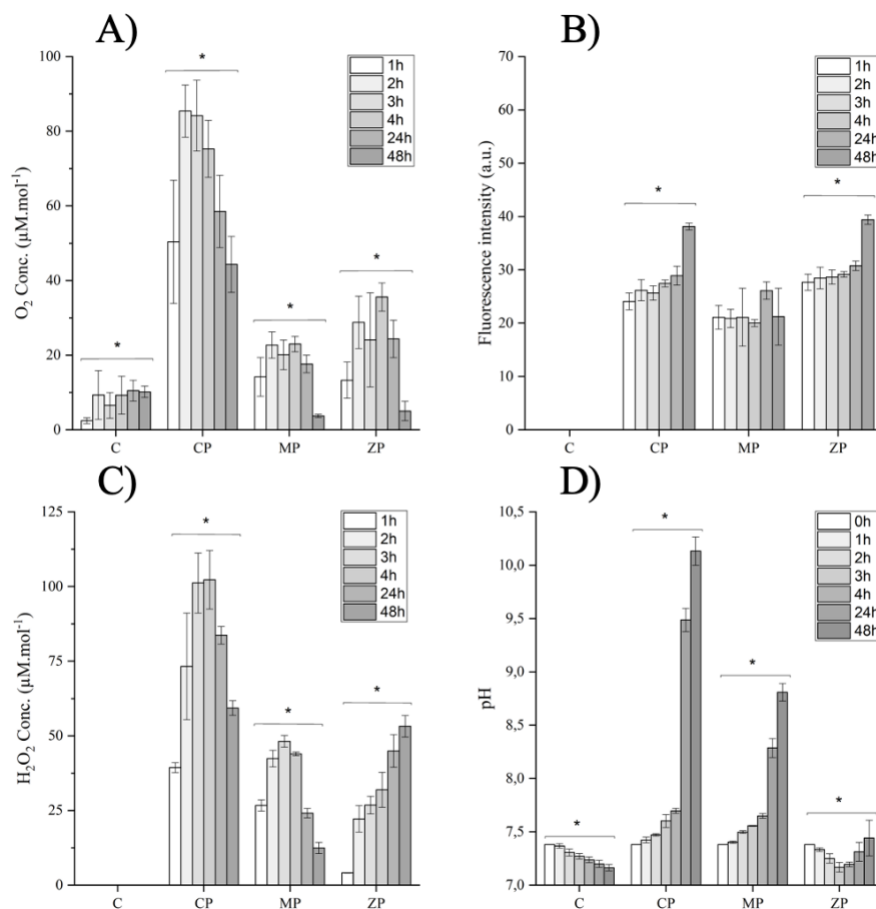


Figure 4.4 O₂, ROS and H₂O₂ concentration over time measured with chemical probe (A)(B)(C). pH of solutions containing samples over time (D). * indicates p < 0.05.

4.4.3 Bacterial activity and cytotoxicity.

The antimicrobial efficacy of the peroxide films against bacteria in solution is shown in Figures 4.5A and 4.5B for *S. Aureus*, a Gram-positive bacteria, and *P. Aeruginosa*, a Gram-negative bacteria, respectively. The results show that the metal peroxides doped samples have strong effects against *S. Aureus* nor *P. Aeruginosa*, while control has no effect at all. The results indicate that the effects were sustained over several days as the concentration of bacteria decreased with time. The calcium peroxide-based samples had the strongest effects on the first day and by the second day no bacteria were detectable. MP had a similar effect but was less effective especially for *S. Aureus*. Initially ZP had a relatively weak effect on the first two days but on the third day a significant (P<0.05) decrease in concentration occurred.

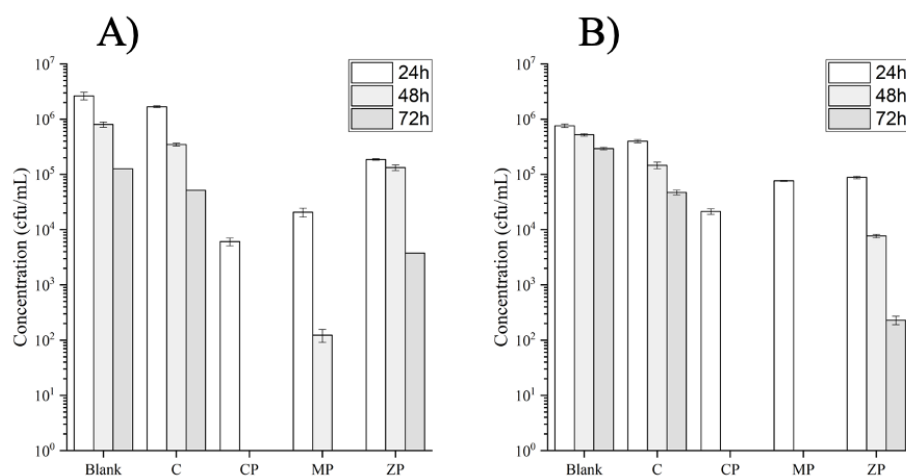


Figure 4.5 Calculated bacterial concentration for *Staphylococcus Aureus* (A) and *Pseudomonas Aeruginosa* (B) with plate count assays. The results were all statistically different, between each timepoint and each sample.

The cytotoxicity on fibroblasts was evaluated to determine if the peroxides were in anyway selectively toxic against prokaryotic cells or had any compatibility with eukaryotic cells (**Figure 4.6**). The results show that the metal peroxides have a negative effect on viability when compared to the control film. Moreover, the viability continues to decrease with time for each metal peroxides-based sample. It appeared that ZP has the best results around 80-70% followed by CP and then MP.

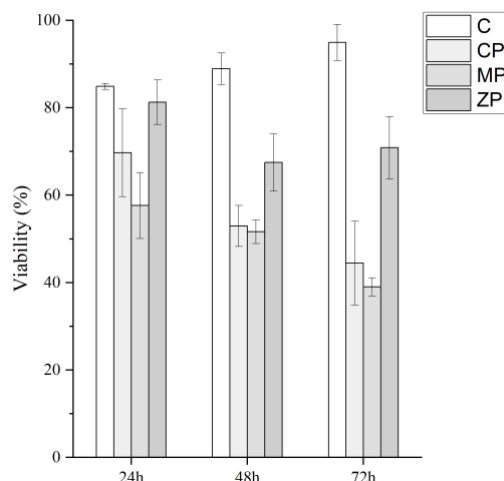


Figure 4.6 Mean % of cell viability from human dermal fibroblasts exposed to different peroxide films after 24h, 48h, and 72h of incubation (n = 3). The color-coded columns represent the film composition tested.

4.5 Discussion

A large body of literature focuses on the use of polymers as diffusion barriers to retard peroxide decomposition reaction rates for sustained oxygen release and also as protective barrier to prevent cell and tissue damage, but no study has compared the efficacy of decomposition products of inorganic peroxides with regards to their antibacterial performance. This study compared biologically relevant peroxides, CaO_2 , ZnO_2 and MgO_2 with different relative solubilities and basicity. The capacity of the polymeric film to absorb and retain water is critical parameter for assessing its character as a diffusion and protective barrier. PCL has shown potential in wound healing applications due to its very stable structure in aqueous state and the incorporation of peroxides seemed not to affecting its morphology or swelling behaviour (Figure 4.3B). The extremely low swelling index of peroxide doped PCL films is attributed to the hydrophobic characteristic of PCL polymers. Given that PCL is a hydrophobic semicrystalline polymer, it is clear that the swelling of the samples was low and that only a small amount of peroxides will degrade after 24h. Control films had a higher swelling degree than peroxide films, and despite their low porosity, water diffusion through MP films appeared to be similar to ZP films and more than CP films

The antimicrobial activity of the degradation product release of the films were determined by measuring the bacterial concentration with plate count assays. The results of film samples are

shown in Figure 4.5. It should be noted that all the samples containing peroxide in our work had activity against the *S. aureus* and *P. Aeruginosa* bacteria, since no decrease in bacterial count was seen for the blank and control films. CP appeared to have a stronger bactericidal effect than MP and ZP. When calcium peroxide reacts in water, they generate Ca^{2+} , O_2 , and H_2O_2 . The antibacterial property of CP is usually associated with the release of H_2O_2 upon degradation [117], which is a well-known compounds to damage bacterial cells and inhibit their growth [131]. In Figure 4.4 C, it can be clearly seen that H_2O_2 concentration increased with time and was highest for the CP film. Furthermore, the surrounding pH related to the ionic dissociation causing an alkalinisation of the environment, was higher for CP and also is known to play a key role in the antibacterial efficacy [132]. The release of Ca^{2+} ions from calcium peroxide has been reported to elicit an antibacterial effect [133]. The generation of ROS which has been found to improve biofilm degradation and appeared to be similar between the CP and ZP [134], but the antibacterial effectiveness of ZP was lower than CP and MP. Consequently, ROS appears to be not a key player rather the combination of pH and hydrogen peroxide. Another product of interest is the release of metal cations, i.e. Ca^{2+} , Zn^{2+} and Mg^{2+} ions, and its nature. Several studies have investigated the antibacterial effects of magnesium, calcium, and zinc cationic metals. The most studied cations so far have been Zn^{2+} ; it has been found to have a strong antibacterial effect on *Streptococcus mutans* and *Actinomyces naeslundii* [135] and to prevent bacterial adhesion by interacting with polysaccharides [136]. Ca^{2+} is a critical actor in bacterial physiology, it has shown to promote bacterial movement, survival reactions, bacterial aggregation and biofilm formation via cationic bridging and attractive acid-base interactions [137], but we are aware of only one study suggesting that Ca^{2+} augments the amount of damage to bacteria during disinfection [138]. To enhance the bactericidal property of Ca^{2+} , mixing Ca^{2+} with Zn^{2+} was found to be an attractive solution as it offered higher antibacterial activity against *Staphylococcus aureus* than calcium films [139]. Mg^{2+} ions have been found to have antibacterial properties, but also to independently inhibit biofilm formation in *Bacillus* species by downregulating matrix genes expression [140]. Overall, we posit that the initiale antibacterial effects caused by metal peroxides are principally due to the degradation product H_2O_2 , and the pH of the environment and the effect of the metal cation on the bacteria, but appears later to strongly depends on the nature of the ions. Given the contact of dressings with open wounded tissue, biocompatibility should be considered. As expected, the control film did not exhibit obvious cytotoxicity, thus confirming that PCL offered a good cytocompatibility. But, peroxide-based films

clearly confirmed that the addition of CaO_2 and MgO_2 within the film did affect significantly the cytotoxicity towards mammalian cells after 48h (30%-40% decrease compared to control), presumably because their H_2O_2 release and pH elevation, despite the addition of calcium dihydrogen phosphate monohydrate. ZnO_2 based films exhibited over 70% cell viability at every timepoint, hence showing minimal cytotoxicity. Further studies are needed to evaluate the specific effects of metal peroxides on fibroblast viability and function. Given that bicarbonate buffer system plays a central role in pH buffering in the human body, cytotoxicity associated with pH alkalinity might not be observed *in vivo*.

4.6 Conclusion

In this study, we successfully developed peroxide-doped polymeric films with promising potential for wound healing and antibacterial applications. The incorporation of calcium, magnesium, or zinc peroxide into a biocompatible polymeric film resulted in efficient prevention of the growth of both Gram-positive and Gram-negative bacteria. The films also demonstrated sustained release of reactive oxygen species and oxygen, contributing to their prolonged antibacterial activity. *In vitro* experiments utilizing human dermal fibroblasts showed a moderate degree of biocompatibility towards fibroblasts. The unique combination of antibacterial efficacy and mammalian cell biocompatibility makes these films a promising advancement for various medical applications, including wound dressings, coatings, and other healthcare products. Further studies involving *in vivo* evaluations are warranted to fully explore the translational potential of peroxide-based materials.

4.7 Author Contributions

Conceptualization, D.J., J.B. and G.M.; methodology, D.J., J. M, C. D, B. H; validation, D.J., J. M, C. D, Y. R; formal analysis, D.J., J. M, C. D; resources, J.B, G.M.; data curation, D.J., J. M, C. D; writing—original draft preparation, D.J; writing—review and editing, J.B and G.M; supervision, G.M.; funding acquisition, G.M and J.B. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 5 GENERAL DISCUSSION

The aim of the thesis was to develop an antibiotic-free material for wound healing. To this end, three different metal peroxides (Calcium, Zinc and Magnesium peroxide) were selected as antibacterial agents and active oxygen sources.

One of the first challenges was to control the release of oxygen and ROS/ H_2O_2 in order to avoid concentration peaks and spread the effects over several days. To this end, the choice of PCL as a hydrophobic barrier was effective in limiting the degradation of metal peroxides in aqueous media with very low swelling indexes, with a maximum of $\sim -6\%$ for magnesium based-sample. In addition, the choice of PCL is supported by the fact that the peroxide-free sample has very low cytotoxicity according to the results of tests on fibroblast cells. The results of the agar plate count tests confirmed that metal peroxides could have a substantial antibacterial effect, since each sample showed a lower bacterial concentration than the peroxide-free control. Another aim of this research was to find a link between the different releases and antibacterial effects, as well as cytotoxicity. Measurements of pH, oxygen concentration, ROS and H_2O_2 showed that the effect of ROS was negligible compared to H_2O_2 and pH for antibacterial effects. However, an unstudied factor is metal ion release while according to the literature, antibacterial effects may also depend on the nature of the metal cation. The calcium peroxide-based sample showed the strongest antibacterial effects, followed by magnesium and zinc. Finally, as these materials are intended for use as wound dressings, it was crucial to inspect the effects of the samples on fibroblast. Despite the addition of pH buffer and iron oxide as catalyst, the results showed that the samples were cytotoxic compared with the control. However, further *in vivo* studies are required to explain these results. In particular, we believe that the effects on pH *in vivo* and *in vitro* should be very different due to the pH buffer system of the human body. The cytotoxicity of samples would then be largely due to H_2O_2 releases.

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS

In conclusion, polymeric oxygenating antibacterial metal peroxides -based dressing have been developed to support the healing of chronic wounds. This is the first comparison to our knowledge of three metal peroxides: calcium peroxide, zinc peroxide and magnesium peroxide for wound healing. The data confirmed that metal peroxides could be used to limit bacterial infections while providing oxygen. CP-based sample significantly reduced *S. Aureus* and *P. Aeruginosa*, after 48 hours the bacteria were not detectable. This sample also released oxygen throughout the 48 hours of the study, with a peak concentration of $80 \mu\text{M.mol}^{-1}$ at the second hour compared with $10 \mu\text{M.mol}^{-1}$ for the control. However, its effects on pH, with measurements of around 9.5 after 24 hours and more than 10 after 48 hours, as well as its rapid hydrogen peroxide release, which is twice as high as the other peroxide-based samples, with up to $100 \mu\text{M.mol}^{-1}$ after 4 hours, make it high cytotoxic. However, both MP and CP have similar viability with non-significant differences at each time point, with approximately 65%, 55% and 40% viability at 24, 48 and 72 hours, respectively. MP, with their lower oxygen release and antibacterial effects, exhibit less interest compared to CP. ZP offered a higher viability (80% at 24 hours and 70% at 48 and 72 hours) but poor antibacterial performances due to its release profile with limited pH variation and a poor hydrogen peroxide release (continuous release of $50 \mu\text{M.mol}^{-1}$ over the 48-hour measurement period). No ideal material could be identified. Although CP-based film could provide a sustained oxygen release and strong bactericidal properties, it also exhibited strong cytotoxicity, whereas ZP offered fairly good viability, but poor antibacterial performance and oxygen release. Given the outcomes of the study, more research is needed. As future work, the study could be extended to a longer period, and different concentrations of ZP could be investigated to further balance the antibacterial effect without impacting cytotoxicity. Additionally, it would be interesting to combine both ZP and CP in the same film to support the weak oxygen release of ZP.

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