

**Antimicrobial activity of silver and copper
nanoparticles incorporated with biodegradable
polymeric scaffolds for wound healing application**

By

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Declaration

I declare that this thesis “Antimicrobial activity of silver and copper nanoparticles incorporated with biodegradable polymeric scaffolds for wound healing application” was my own work, carried out under the supervision of Prof N. Moloto, Dr M. Mlambo, Dr P. Tetyana and Dr Z. Njengele-Tetyana. This work is being submitted for the degree of Doctor of Philosophy (chemistry) to the School of Chemistry, Faculty of Science, University of the Witwatersrand, Johannesburg, and has not been submitted before for any degree or examination at any university.

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Abstract

There has been a significant increase in the prevalence of chronic diseases such as diabetes and cancer which cause wounds like diabetic foot ulcers and pressure ulcers. This usually causes infection(s), leading to the amputation of limbs or even death. Chronic wounds do not follow the typical stages of wound healing and thus either take a very long time to heal or do not heal at all. Therefore, the need for developing advanced and novel wound treatment products that offer infection control and sharp debridement is vital.

To combat this issue, this project focussed on fabricating bioactive nanomaterials to design an advanced wound dressing in the form of 3D scaffolds. The scaffolds were made of nanocomposites containing biocompatible and biodegradable polymers decorated with antimicrobial active Cu and Ag NPs. Prior to designing the scaffolds, pristine Ag and Cu NPs were fabricated and their antimicrobial activity and cytotoxicity were tested. The antimicrobial activity of these NPs was tested against *E. coli* and *S. aureus* bacterial strains, while their cytotoxicity was evaluated using baby hamster kidney fibroblasts (BHK-21) cells.

Ag NPs with various sizes and spherical shapes were synthesized using dopamine (DA) as a reducing agent. Ag NPs with the smallest diameter exhibited the most antimicrobial activity compared to those with big diameters. The cytotoxicity of Ag NPs was very low, with cell viability of over 70% between 2 $\mu\text{g/mL}$ and 32 $\mu\text{g/mL}$. At concentrations above 32 $\mu\text{g/mL}$, a decrease in cell viability was observed, indicating increasing toxicity, especially for small NPs. Cu-based NPs were also synthesized using hydrazine and DA as reducing and capping agents, respectively. Pure metallic Cu, Cu₂O and the mixture of Cu and Cu₂O (Cu/Cu₂O) NPs were separately prepared. Cu and Cu₂O NPs exhibited promising antimicrobial activity against *E. coli* and *S. aureus*. An improved antimicrobial activity was observed for Cu/Cu₂O NPs, compared to the Cu and Cu₂O NPs, and this was attributed to the synergistic effect. The cytotoxicity of Cu₂O NPs was higher compared to that of Cu and Cu/Cu₂O NPs.

The antimicrobial activity and cytotoxicity of both Ag and Cu NPs incorporated with polydopamine (PDA) were also studied. Cu and Ag NPs were either embedded within the matrix of PDA (Cu-PDA or Ag-PDA) or incorporated on the surface of PDA (Cu@PDA or Ag@PDA). Cu@PDA and Ag@PDA showed stronger antimicrobial activity than Cu-PDA and Ag-PDA due to the NPs exposure to the bacteria. These nanocomposites showed very

low cytotoxicity with cell viability of over 80 % at concentrations as high as 250 µg/mL. However, a drastic decrease in cell viability was observed when the concentrations of Cu@PDA and Ag@PDA were increased above 250 µg/mL. Taking advantage of the strong biological activity exhibited by Cu and Ag NPs, bimetallic nanoalloys of these two metals were prepared. Nanoalloys with a fibrous shape were obtained as bimetallic nanofibres (BNF). The mole ratio of Cu:Ag was found to have an effect the antimicrobial activity of the BNFs. The BNFs prepared with a mole ratio of 1:2 (BNF-2) exhibited a strong antimicrobial activity compared to 1:1 (BNF-1) and 2:1 (BNF-3). Furthermore, the BNFs showed minimal cytotoxicity towards the BHK-21 cells at low concentrations (7.8 µg/mL), with the cell viability ranging between 75-90 %. Compared to BNF-1 and BNF-2, BNF-3 was found to have a negative impact on cell viability when the concentrations were increasing.

The novel and porous 3D scaffolds were prepared by coating chitosan/gelatine (CS/Gel) scaffolds with PDA using the freeze-drying method. This was followed by decorating the surface of the scaffolds with either Ag or Cu NPs, *in situ*. The presence of the catechol functional group inherent from PDA facilitated the reduction of Ag ions to form pure zerovalent Ag NPs on the surface of the scaffolds. However, to reduce Cu ions to form Cu NPs, an access reducing agent (hydrazine) was added. The scaffolds showed reasonably high fluid uptake (FU) with time-dependent biodegradability, a property that would be advantageous in controlling wound exudates. These scaffolds also showed excellent antimicrobial activity against *E. coli* and *S. aureus* with low cytotoxicity towards human fibroblast cells. Taken together, the designed scaffolds could act as a barrier from the external environment, help prevent bacterial infections and further accelerate skin cells regeneration. The most important feature of these scaffolds is their potential ability to penetrate deep wounds and provide a conducive environment for skin cells to regenerate without any difficulties from bacterial colonization.

Dedication

This work was dedicated to my mother (Nyana Johannah Mulukwane) and father (Mbulaheni Josias Shumbula)

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List of abbreviations

Ag	Silver
AgNPs	Silver nanoparticles
Cu	Copper
CuNPs	Copper nanoparticle
NPs	Nanoparticles
DA	Dopamine
PDA	Polydopamine
Ag@PDA	Silver nanoparticles on the surface of polydopamine
Ag-PDA	Silver nanoparticles embedded with polydopamine
Cu@PDA	Copper nanoparticles on the surface of polydopamine
Cu-PDA	Copper nanoparticles embedded with polydopamine
CS	Chitosan
Gel	Gelatin
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>E. coli</i>	<i>Escherichia coli</i>
MIC	Minimum inhibitor concentration
MBC	Minimum bactericidal concentration
CC50	Cytotoxic concentration at 50 % cell viability
BHK-21	Baby hamster kidney fibroblasts cells
Au	Gold
PVA	Poly(vinyl alcohol)
PVP	Poly(vinyl pyrrolidone)
PEG	Poly(ethylene glycol)
FTIR	Fourier-Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
PXRD	Powder X-ray Diffraction
TGA	Thermogravimetric analysis
DTA	Derivative thermal analysis
XPS X-ray	Photoelectron Spectroscopy
BE	Binding energy
SPR	Surface plasmon resonance

UV-Vis	Ultraviolet-visible spectrophotometry
mL	millilitre
nm	Nanometres
µg	microgram
g	Grams
mM	millimolar
h	Hour(s)
min	Minute(s)

Chapter 1

General background

1.1 Introduction

Human skin is the body's heaviest and largest organ, covering about 6 m. It has a thickness of about 1.5 to 4 mm depending on the body region and ageing. It plays a major role in forming self-repairing and renewing interfaces between the body and the surrounding environment. It protects the internal body against microbial invasion and further prevents mechanical, chemical, osmotic, thermal and photodamage. The skin is permeable and can absorb and excrete useful and wasteful chemical substances, respectively. In general, skin is a protective barrier that helps the body to maintain its internal temperature (homeostasis) and protect against various infections [1-5]. Disruption of skin tissue can lead to bacterial or viral infections or permanent damage to the skin if not treated [2]. Wounds are one of the most common clinical challenges, with complications that result in a constant cause of morbidity and mortality [6, 7]. Recently, therapeutic advances and new technology development have proven to be promising solutions for acute and long-term wound management [8, 9]. The high rate of wounds occurrence as a result of injuries or infections together with their tremendous effect on the ageing population remains one the most social and economic impacts worldwide. Adding to many acute wounds caused mostly by injuries, there is a countless number of chronic wounds, which are highly associated with diseases such as cancer, diabetes and HIV/AIDS; and are challenging to manage or heal. It has been estimated that 120 in 100 000 people between the ages of 45 and 65 years get affected by chronic wounds every year, while 800 of them over 75 years get affected too [6]. Furthermore, acute wounds can advance to chronic wounds as a result of complications during the healing process. This can be due to mismanagement of wounds leading to bacterial infection hence taking time to heal.

Nanotechnology is an advanced field that brought innovations that are improving everyday life. Through nanotechnology, materials that are useful in various applications such as catalysis, electronics, drug delivery and wound healing therapeutics have been developed [10-13]. This is because nanomaterials possess characteristics and properties that cannot be assumed in their bulk forms. The most used nanomaterials include but are not limited to

polymers and metal nanoparticles (NPs). Metal NPs have been utilized in various scientific and engineering fields due to their intrinsic properties. The prevalence of multidrug-resistant bacteria has seen an increased use of metal NPs in biomedical applications such as wound healing [14-16]. Amongst other metal NPs, silver (Ag) and copper (Cu) have attracted great attention in wound healing applications. This is because Ag and Cu NPs have shown a great range of antimicrobial spectrum which can solve the long-standing problem of bacterial infections in wounds [17-19]. As a result, these NPs have also been incorporated in polymer matrices to enhance their antibacterial and wound healing activity. The synergy that comes from incorporating Ag and Cu NPs in biodegradable polymers have a very promising effect on wound healing [20-22]. Biodegradable polymers such as chitosan and gelatine are very essential in wound healing. These polymers can be designed into three-dimensional (3D) scaffolds that can be used to treat deep chronic wounds through tissue regeneration [23-25]. In this project, antimicrobial active Ag and Cu NPs were synthesized using dopamine as a capping and reducing agent. The NPs were also incorporated with biodegradable polydopamine (PDA), chitosan (CS) and gelatine (Gel). The NPs decorated 3D scaffolds made of CS and Gel coated with PDA were also designed. The antimicrobial activity of the designed materials was investigated against gram-positive and gram-negative bacterial strains. Furthermore, the cytotoxicity of the materials and cell attachment on the surface of the scaffolds were investigated using fibroblasts cell lines to test the potential in wound healing.

1.2 Wounds and wound types

Wounds are defined as disruption of skin tissue or normal anatomical structure and functions of skin [26]. They can range from just a minor break of skin epithelial integrity to a deeper break into subcutaneous tissue that can result in damage to other structures such as muscles, nerves, tendons and even bones. Furthermore, a wound can arise from the malfunctioning of other body organs as a result of diseases. Irrespective of the form and the cause of the wound, skin tissue gets damaged and the local environment within it gets affected, mostly through bacterial infections. The body's response towards a wound involves bleeding, contraction of vessels, followed by coagulation and inflammatory response [27, 28]. However, wound healing is a dynamic and complicated process involving several overlapping phases. Regardless of its complexity, the process of wound healing begins immediately after the moment of injury. It involves both local and migratory cell populations, extracellular matrix and the action of soluble mediators. The phases underlying the wound healing process which

will be explained later include (i) haemostasis; (ii) inflammation; (iii) proliferation; (iv) maturation; and (v) remodelling. These phases proceed continuously throughout the repairing process [27, 29-32].

The time it takes for the wound healing process to complete can vary depending on the body region or ageing. This process can be diverse and may take several months or years to complete. A wound is completely healed when the anatomical structure, functions and physical appearance of the skin tissue have returned to normal. Even though most wounds occur from simple injuries, some wounds do not heal adequately with time, which can lead to most complicated wounds that do not heal at all [27, 33].

1.3 Classification of wounds

Skin disruption or trauma can be categorized into various degrees: A disruption that only happens in the epidermis layer of the skin; this is the small disruption of the skin (mainly caused by cuts or small injuries) that results in an acute wound, which can simply heal through re-epithelialization without any tissue transplantation. The severe disruption of the skin is the one that results in the damage of both the dermal and subdermal tissues [6]. This damage results in a chronic wound, which most of the time does not heal adequately as a result of a deep dermal injury. In most cases of ulcers and burn wounds, cell regeneration is compromised except the edges of the wound. As a result, re-epithelialization takes long and is complex because of the base scarring [6-8].

1.3.1 Acute wound

Acute wounds are mostly caused by traumas and usually proceed by following an orderly and timely healing pathway. These wounds repair themselves and usually take 8 to 14 weeks to heal, with the result of both anatomically and functional restoration of skin tissue. Acute wounds can also be caused by mechanical damage induced by sheer, blunting, corrosive chemicals, irradiation and/or surgical procedures. Care for acute wounds mostly depends on the severity of the wound [27, 32-35].

1.3.2 Chronic wound

Wounds that fail to succeed through the normal phases of the healing process and cannot be healed in an orderly and timely manner are classified as chronic wounds. Unlike acute wounds, these wounds take more than 14 weeks to heal and could relapse [9, 36]. The functional and anatomical structure of skin tissue becomes poor since the healing process in

chronic wounds proceeds incoherently. The complexity of the healing process in the chronic wound is governed by factors such as infection, exudates, necrosis and hypoxia. These factors prolong one or more phases (haemostasis, inflammation, proliferation or remodelling) associated with wound healing. Chronic wounds may result from burns, pressure, arterial and venous insufficiency or specific diseases such as tumour, diabetes, and cancer [37-39].

1.4 Phases of wound healing

The process of wound healing is multifunctional, complicated and it involves molecular, anatomical and physiological phenomenon. Although this process is continuous and complicated, it is arbitrarily divided into four critical phases: homeostasis, inflammatory, proliferation and tissue remodelling, which are very cohesive and overlapping [7, 9]. These phases aid the understanding of the physiological processes that take place in wounds and surrounding tissues as shown in **Figure 1.1**.

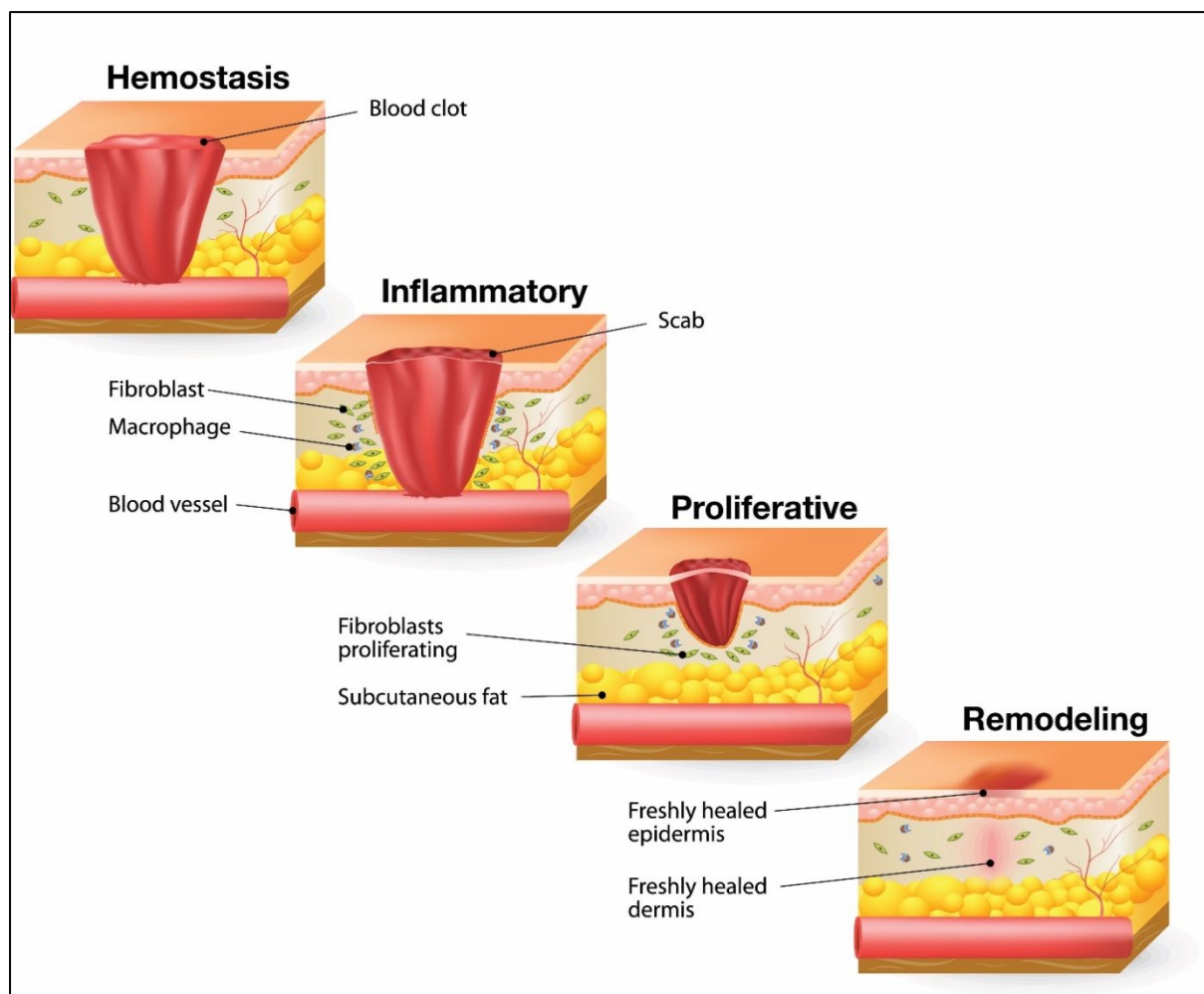


Figure 1.1: The phases governing the wound healing process [40].

1.4.1 Haemostasis

Haemostasis is the initiating phase and the foundation of the healing process. The significance of this phase is to reduce or prevent draining of blood or exsanguination. This phase helps to protect the circulatory system so that other organs of the body will continue to operate despite the injury. Moreover, the haemostasis phase provides the organisational structure of invading cells that are needed in the later phases of the process. This phase is governed by the balance between endothelial cells, thrombocytes, coagulation, and fibrinolysis which further promote the deposition of fibrin at the wound thus influencing the repairing processes [29-32].

1.4.2 Inflammation

The main function of the inflammatory phase is to initiate the protection against microbes. It begins at the early stages of coagulation whereby various molecular events are activated. This includes the infiltration of wound site by neutrophils, thus reducing or preventing infections. Bacteria, damaged tissues and other foreign particles are destroyed and removed by phagocytosis which is the main task brought about by neutrophils. Neutrophils start to infiltrate the wound site two days after the injury. As a result, this phase lasts for about five days of injury and is characterized by the redness, heat and swelling of the wound site [8, 27, 41].

1.4.3 Proliferation

This phase is initiated about two days after injury and last for about two weeks. It generally overlaps with the inflammatory phase and is characterized by epithelial proliferation and migration over the provisional matrix within the wound; a process known as re-epithelialization. In this phase, the newly formed granulation tissue comprising of collagen and extracellular matrix (ECM) ‘rebuild’ the wound and further develop a new network of blood vessels. During repair, the dermis, fibroblast and endothelial cells promote the growth of capillaries and the formation of granulation tissue together with the collagen at the wound site. Collagen and proteoglycans are produced at the wound bed by the fibroblasts, which are the main components of the ECM [42-45].

1.4.4 Tissue remodelling

The remodelling is the final phase of the wound healing process following the robust proliferation phase and ECM synthesis. This phase can last for years depending on the type

and the complexity of the wound. It is responsible for the formation of new epithelium and final scar tissues [46, 47]. The newly formed capillaries regain their strength so that the vascular density returns to normal. The tensile strength of the wound increases progressively in parallel with collagen collection [48, 49]. Collagen fibres may regain approximately 80% of the original strength compared with unwounded tissue. The acquired final strength depends on the localization of the repair and its duration, but the original strength of the tissue can never be regained [50].

1.5 Problem statement

Wound recovery/healing is a critical and complicated biological activity in the body and involves four precisely, highly and overlapping programmed phases mentioned before. For a successful process of wound healing to occur, all these phases must take place in an actual sequence and timeframe. Disruption of one or two of these phases can result in the formation of a chronic wound: a wound that cannot heal in a regular set of stages and within an expected amount of time. Several factors can impede these phases, thus compromising the wound healing process. These factors include age, sex hormones, nutrition, stress, diseases or bacterial/viral infections to name a few. For instance, diabetes mellitus is commonly known to cause a chronic wound known as diabetic foot ulcer (DFU) which is deadly. DFU are one of the biggest problems around the world due to the prevalence of diabetes mellitus, more especially in developing countries. According to the International Diabetes Federation (IDF), the number of South African adults living with diabetes in 2020 increased to 4.5-million people (12.8% of the SA adult population), more than double the figure estimated in 2017 [51, 52]. About 10-25% of these people will develop DFU at some stage in their lives. This can potentially increase the rate of amputations due to the lack of efficient treatment procedures. It is estimated that about 60.2% of non-traumatic lower-limb amputations in South African public hospitals are due to diabetes. In addition, the number of communicable diseases is rising continuously and may result in higher numbers of chronic wound patients. Therefore, the need for developing advanced and novel wound treatment products that offer infection control and a fast-healing process is urgent [53].

Even though various kinds of wound dressings are currently available for the treatment of wounds caused by diabetic ulcers, burns, split skin graft donor sites and pressure sores, bacterial infections on the wounds remains a formidable problem. This is because traditional wound dressings only cover the wound without protection from bacterial infection. Bacterial

infection compromises the wound healing process and can delay or even prevent a wound from healing. Antibiotics have been used for decades to treat wounds through the prevention of infection. However, antibiotics have proven to possess some complications in the body. These include the emergence of drug-resistance bacteria, liver and renal toxicity. Various nanomaterials and biodegradable polymers have played a significant role as antimicrobial active agents with wound healing properties. The importance of the studies to look into the combination (forming a composite) of these antimicrobial active materials will provide and open more gates for understanding and scaffolding sophisticated wound dressings with the potential to prevent bacterial infection and further improve the healing process. There is a huge call for fabricating and designing advanced wound dressings that can solve the problems associated with traditional dressings.

1.6 Motivation/ rationale

Colloidal NPs are interesting materials that have gained vast attention in the field of science and technology due to their electronic, optical and structural properties. These materials include metal nanostructures (e.g., Au and Ag), metal oxides, and other semiconductors. Ag and Cu NPs exhibit various native size-dependent properties such as surface energy and light scattering or absorption compared to their bulk materials. The ease of synthesizing Ag and Cu NPs and their surface modification with various molecules and polymers in an ambient environment have led to their vast application in photovoltaics, electronics, drug delivery systems, water treatment and wound healing. To the best of our knowledge, Ag and Cu NPs, including antibiotics are the most significant and commonly known antimicrobial agents. Unfortunately, regular consumption of antibiotics may result in an increase in microbial drug resistance, which can further compromise the treatment potency and significant economic problems. Ag and Cu NPs, however, have proven to display outstanding antimicrobial activity as a result of their ability to deactivate respiratory enzymes of bacteria by interfering with DNA replication and disrupting the cell membrane. Even though these materials have been exploited in commercial products, they still attract more research due to their practical application in various fields. In recent decades, the effect of size, shape, surface charge, solution chemistry, and surface coating of these NPs on antibacterial properties have been widely studied. However, the critical problems of aggregation and oxidation of these NPs, especially Cu still exist and limit their practical application. Incorporating Ag and Cu NPs with biodegradable polymers such as chitosan (CS) and gelatine (Gel) can improve antimicrobial activity and wound healing properties due to synergistic effects. Therefore, the

focus of this study was to investigate the antimicrobial activity of Ag and Cu NPs incorporated on the surface of skin tissue scaffolds made of chitosan and gelatine together with their potential application in wound healing. This project further showed, for the first time, the ability of PDA to coat the skin tissue scaffolds made of chitosan and gelatine which facilitated the *in-situ* incorporation of Ag and Cu NPs. Finally, the study showed the potential of the fabricated NPs and the scaffolds in wound healing.

1.7 Research aims and objectives

The main aim of the study was to investigate the antimicrobial activity of Ag and Cu NPs incorporated with biodegradable polymers for application in wound treatment.

Specific objectives of the project include:

- Synthesis and characterization of Ag and Cu NPs.
- Synthesis and characterization of PDA.
- Incorporation of Ag and Cu NPs on the surface of PDA.
- Fabrication of Ag and Cu NPs incorporated chitosan/gelatine tissue scaffolds.
- Antimicrobial activity and the cytotoxicity studies of the synthesized materials towards human skin cells.
- Cell proliferation and cell attachment studies.

1.8 Thesis outline

This thesis consists of nine chapters outlined below. Six chapters were written in the form of manuscripts.

Chapter 1: This chapter gives a brief background on the study, particularly on wounds and the problems associated with them. The rationale of this project was also outlined in this chapter together with the main aim of conducting this study.

Chapter 2: In this chapter, more details on problems associated with wounds are given. This chapter further discusses the recent literature on the current solutions and the fabrication of materials suitable for wound healing.

Chapter 3: This reports on the dopamine assisted synthesis of Ag NPs, their antimicrobial activity and cytotoxicity towards skin cells.

Chapter 4: The effect of incorporating Ag NPs inside and outside PDA on antimicrobial activity and cytotoxicity was investigated in this chapter.

Chapter 5: This chapter discusses and compares the antimicrobial activity and cytotoxicity of dopamine capped Cu, Cu₂O and further investigates the synergistic effect due to Cu/Cu₂O NPs.

Chapter 6: The chapter showcased the incorporation of Cu on the surface and within the matrix of PDA followed by comparing their antimicrobial activity and cytotoxicity towards human skin cells.

Chapter 7: This chapter showcased a new method of fabricating Cu/Ag alloys and investigated their antimicrobial activity and cytotoxicity towards human skin cells.

Chapter 8: In this chapter, the fabrication of tissue scaffolds made of chitosan and gelatine and coated with PDA was discussed. The chapter further showcases the *in-situ* incorporation of Ag and Cu NPs with the matrix of the scaffolds. The antimicrobial activity, cytotoxicity and cell migration studies were showcased in this chapter.

Chapter 9: This chapter provides the summary and the general conclusions of this thesis. It further suggests the recommendations for further investigations.

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Chapter 2

Literature review

2.1 Introduction

Essential and non-essential metals such as copper (Cu) and silver (Ag) are highly lethal to pathogenic microorganisms. These metals have high micro-biocidal activity even at very low concentrations. Due to their micro-biocidal activity, they have been applied as antimicrobial agents in various fields such as agriculture and the healthcare industry for centuries. Ag and Cu, for instance, have been used in the manufacturing of containers to disinfect water and preserve food for long periods [1-5]. Antimicrobial metals have also been used as organic biocontrols in agriculture [6]. In medicine, Cu salts have been used as astringents, while Ag and Ag salts, on the other hand, are being used as sutures and preventing infections in surgical wounds [7-10]. Metals have also been used to treat syphilis, tuberculosis and gonorrhoea [11] [12]. The discovery of antibiotics in the 1920s reduced the attention on metals as antimicrobial agents [1]. However, the development of multidrug-resistant (MDR) bacteria towards antibiotics have caused a drop in the application of antibiotics. This is because MDR bacteria have posed serious health concerns in infectious diseases such as gonorrhoea, pneumonia, tuberculosis and chronic wounds treatment [13-16]. For instance, one of the leading causes of bacterial infections in the healthcare sector, *Pseudomonas aeruginosa* (*P. aeruginosa*), has been reported to have increased resistance to several antibiotics [17-19]. Another example is the resistance of *Escherichia coli* (*E. coli*) towards streptomycin as a result of the bacteria's ribosomal mutation [20, 21]. *Staphylococcus aureus* (*S. aureus*), the main bacteria associated with wound infections, have developed resistance towards trimethoprim and methicillin antibiotics through the inactivation process using specific enzymes [21, 22].

The lack of development of new and effective antibiotics has forced scientists to look into developing alternative antimicrobial agents that are more efficient. Recently, the use of antimicrobial metals in healthcare is undergoing yet another recognition due to the threat posed by these MDR pathogens. Nanotechnology and material sciences have revolutionized antimicrobial metals in biomedical fields. With the developments and advancement in these fields, various improvements and control over properties of antimicrobial active metals such as Ag and Cu have been made. The ability to synthesize and modify the properties of these

metals has resulted in novel materials with improved antimicrobial activity. Nanotechnology has the ability to design metals into nanosized materials. The optical, chemical and physical properties of metals change drastically when they are in nanoscale. Therefore, the use of Ag and Cu as antimicrobial agents has become very fruitful since most pathogenic bacteria have developed resistance against most antibiotics. Ag and Cu NPs are amongst the safe and non-toxic antibacterial agents. In addition, they are known to possess a broad spectrum of antifungal, anti-inflammatory, anti-viral and anti-angiogenic activity [23-29]. Hence, medical and biological applications such as medical devices and wound dressings have improved greatly because of the various properties that these metal NPs can offer.

One of the recent improvements in the antimicrobial activity of metal NPs is to incorporate them on the surface of polymer matrices to form metal/polymer nanocomposites. Incorporating these NPs on the surface of biocompatible polymers and proteins further improves their applicability in wound healing [2, 30-32]. In addition, the metals' stability also improves since NPs in suspension tend to agglomerate quickly, which affects their properties. In metal/polymer nanocomposites for wound healing, polymers support metal NPs for stability while at the same time offering the properties required for the wound healing process. Some polymers are antimicrobial active, which, when incorporated with antimicrobial active NPs like Ag and Cu, promote the synergistic effect of the nanocomposites, which is also advantageous in wound healing [33-38]. For instance, incorporating NPs on or inside the polymer matrix such as polydopamine (PDA), Gel and CS has greatly improved their antimicrobial activity, wound healing properties and reduced their toxicity [38-42]. These polymers have been widely used in wound healing applications due to their intrinsic properties such as biocompatibility, biodegradability and non-toxicity. The ability of these polymers to be synthesized or modified into different shapes opens more opportunities for biological and other applications. PDA, for instance, can be synthesized as solid spheres, hollow spheres or sheets, amongst other shapes. PDA can also adhere to various surfaces due to the presence of the catechol and amine functional groups [43-47]. On the other hand, the Gel can be dissolved in water to form gels that can be modified into fibres or 3D shapes. Gel is composed of peptide sequences which are very important for cell adhesion. In addition, Gel can easily absorb exudates and control a moist environment, thus stimulating the wound healing process [48-52]. Like Gel, CS can also be shaped into fibres, 3D structures, and spherical or irregular-shaped NPs. CS is a positively charged biopolymer with a unique chemical nature dominated by reactive hydroxyl and amino acids. In addition,

chitosan is antimicrobial active, which offers a synergistic effect when incorporated with antimicrobial active metal NPs [53-59]. These polymers, in their 3D structures, also known as scaffolds, have gained a wide interest in wound healing research. Polymer scaffolds incorporated with NPs don't only prevent bacterial infections, they also promote cell adhesion and proliferation, thus enhancing the development of new skin tissue and promoting a fast-healing process [39, 48, 53]. Therefore, there is a call to develop novel bioactive scaffolds that can easily penetrate and improve the rate of the deep chronic wound healing process. The focus of this study was to fabricate biodegradable polymer scaffolds incorporated with antimicrobial active Ag and Cu NPs for potential application in wound healing. More literature on antimicrobial activity of metal NPs and biodegradable polymers and their application as skin tissue scaffolds was done and reported in sections below.

2.2 Metal nanoparticles

2.2.1 General aspect on the synthesis and properties of metal nanoparticles

During the last two decades, various nanomaterial synthesis methods have been implemented, fulfilling high expectations of nanotechnology. The demand for nanomaterials in various scientific fields has contributed to the progress of nanofabrication. The use of metal NPs in different biological areas such as diagnostics, drug delivery and therapeutic devices has become of paramount importance [60]. NPs can be synthesized using top-down or bottom-up synthetic approaches, as shown in **Figure 2.1**. A top-down approach involves using nanofabrication tools rolled by external experimental parameters to design a structure with desired shapes and properties, starting from bulk materials and reducing them to nanoscale [61] [62]. A top-down approach includes lithography, chemical etching, laser ablation, and ball milling methods. Some of these methods offer the advantage of fabricating monodispersed NPs with consistent morphologies and sizes. However, these methods are usually costly due to sophisticated instrumentation requirements and typically result in low product yields [63-69]. The bottom-up approach, also known as self-assembly, involves assembling basic units, i.e., atoms and molecules, into clusters to produce a nanoscale structure [61, 70]. For instance, a monomer molecule can be used as a building block to synthesize organic or polymer NPs through emulsification, precipitation or self-assembly. Nucleation and growth are the main processes associated with the synthesis of metal or inorganic NPs. Methods that apply the bottom-up approach include chemical reduction, vapour phase deposition, sol-gel and spray pyrolysis, amongst others [71-76]. This approach

is extremely favourable due to the easy control of the nanostructures' sizes and shapes with remarkable uniformity.

Varying reaction parameters can alter the properties of NPs during synthesis. For example, changing the concentration of starting materials during synthesis can significantly affect the size of NPs. Furthermore, adding a surfactant or a capping agent can influence the shape of the NPs. Varying sizes and shapes of NPs have been reported to have a great influence on their properties which is very significant during their applications [78, 79]. NPs with sizes ranging from 1 nm to 100 nm with different shapes such as a sphere, sheets, stars, rods and flowers have been synthesized before. The influence of sizes and shapes of NPs in catalytic, optical, and biological properties have been comprehensively investigated in literature [80-84]. Therefore, it is believed that the development in the novel fabrication of nanomaterials will lead to a massive transformation in advanced technology in biomedical areas and others. The development of novel nanomaterials with varying properties can be a great solution to the current global health crisis.

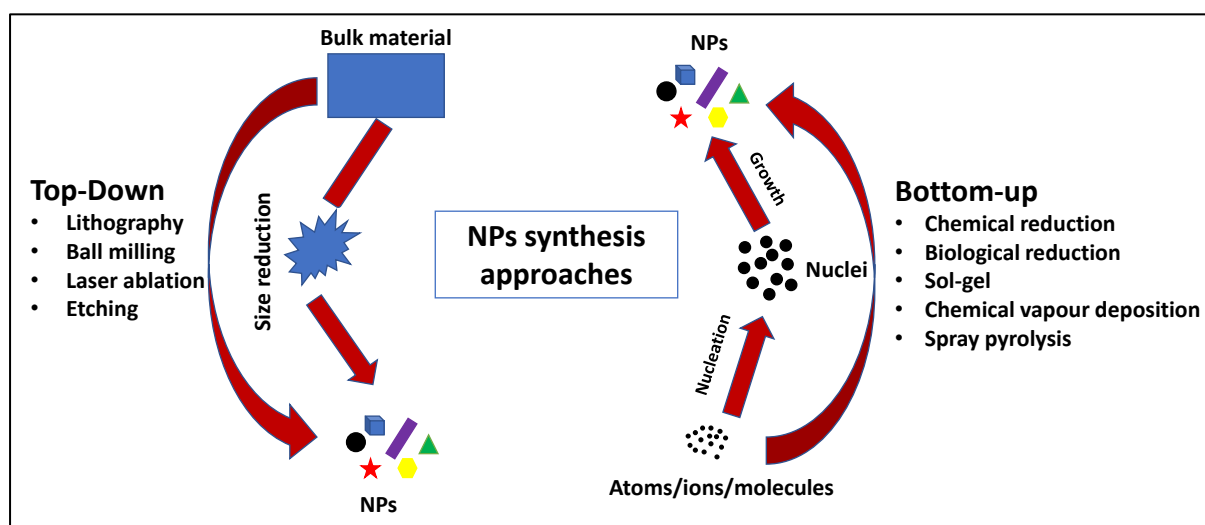


Figure 2.1: Bottom-up and top-down approaches for synthesizing NPs.

2.3 Antimicrobial activity of metal nanoparticles

Over 100 000 tons of antibiotics are produced every year. However, MDR bacteria still pose serious health issues around the world. Over 70% of the infections caused by bacteria are resistant to at least one of the antibiotics produced to treat them. This resistance is mainly caused by the ability of bacteria to mutate and adapt, which results in them gaining resistance

against antimicrobial agents through inactivating or reducing their efficacy [85-89]. Due to this alarming global health crisis, research on developing alternative antimicrobial agents became a topic of interest. Metal NPs have gained broad interest as antimicrobial agents in various fields, including water treatment, food packaging and biomedical, owing to the advancements in nanoscience and nanotechnology [90-92]. Metal NPs can extend the antimicrobial activity and are also able to interact and inactivate MDR bacteria due to their small sizes and high surface to volume ratio. The interaction of metal NPs with the cell surface of bacteria is the main important feature that induces their activity [88, 89].

Bacteria are classified as either gram-negative or gram-positive, and it is crucial to understand the distinction between their cell walls. The cell walls of both gram-positive and gram-negative bacteria are negatively charged. Gram-positive bacteria have a thick peptidoglycan layer made of N-acetylglucosamine and N-acetylmuramic acid cross-linked by amino acids and have no outer lipid membrane. In contrast, gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane with high lipids and liposaccharides content. The high level of liposaccharides in gram-negative bacteria is the main contribution to their high negatively charged cell wall [93-95]. Gram-negative bacterial strains include *Pseudomonas aeruginosa* (*P. aeruginosa*), *Helicobacter pylori* (*H. pylori*) and *Haemophilus influenzae* (*H. influenzae*), which are respectively resistant to carbapenem, clarithromycin and ampicillin [96-98]. Gram-positive bacteria include *S. aureus*, *Enterococcus faecium* (*E. faecium*) and *Streptococcus pneumoniae* (*S. pneumoniae*) which are resistant to methicillin, vancomycin and penicillin, respectively [99-101]. Gram-negative bacteria are more sensitive to antimicrobial active metal NPs than gram-positive bacteria. This is because of their thin peptidoglycan layer and highly negatively charged cell walls. Metal NPs have various mechanisms of action through which they act against bacteria to fight most MDR bacteria. They easily bond with the negatively charged cell walls due to an electrostatic interaction, which results in the disruption of the wall and increases permeability. After penetrating the bacterial cell, NPs can interact or release free ions that interact with vital organelles (Proteins and DNA). In addition, the ability of metal NPs to produce reactive oxygen species (ROS) induces oxidative stress that suppresses the bacterial antioxidant defence [102]. Shown in **Figure 2.2** is a diagram demonstrating various ways that metal NPs use to immobilize bacteria.

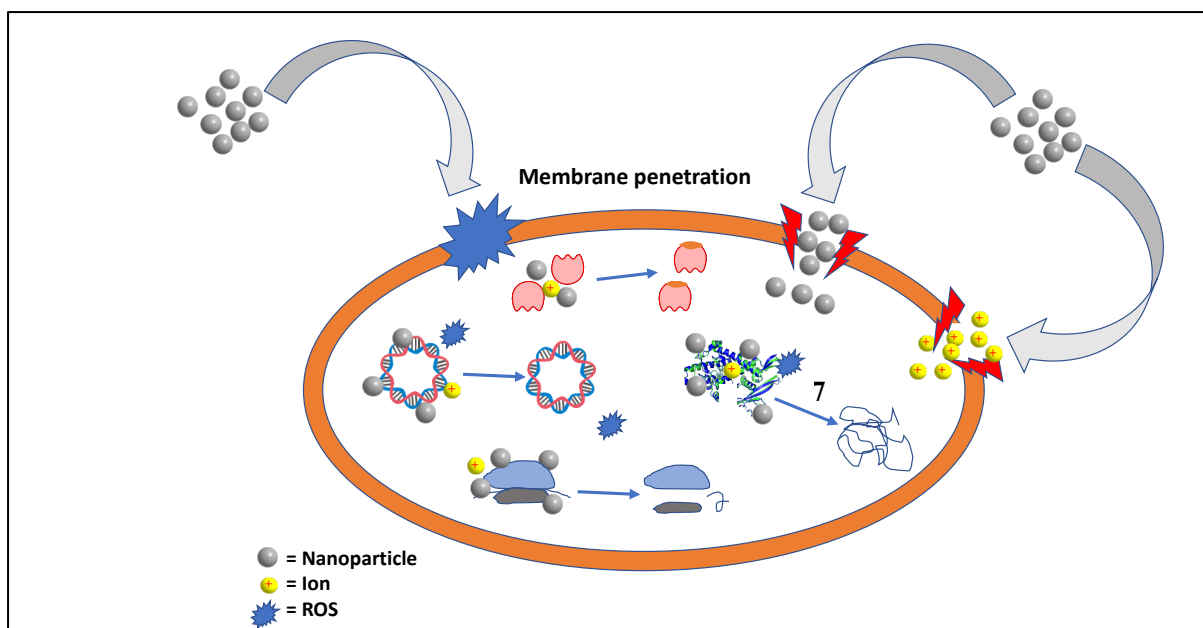


Figure 2.2: General mechanism of action of metal NPs against bacteria.

Some of the widely used metal NPs as antimicrobial agents include, Ag, silver oxide (Ag_2O), Cu, copper oxide (CuO), titanium dioxide (TiO_2), zinc oxide (ZnO) and gold (Au) [103-109]. Hernández-Sierra *et. al.* reported comparative investigation of the bactericidal activity of AgNPs, ZnO, and Au on *Streptococcus mutans* (*S. mutans*). Their results indicated that Ag nanoparticles exhibited the most activity for controlling *S. mutans*. The antimicrobial activity of these NPs is dependent on various factors, including their shapes, sizes and the methods used to prepare them. For instance, NPs with small sizes are known to have a great antibacterial spectrum compared to those with bigger sizes [110]. It has also been reported that NPs synthesized with various plant extracts possess good antimicrobial activity due to the synergistic effect offered by the combination of intrinsic properties of the NPs themselves and the active compounds from the plant used [105].

2.3.1 Antimicrobial activity of silver and copper nanoparticles

Ag and Cu NPs are potential candidates for solving problems associated with diseases caused by bacterial infections. In addition, these NPs have been reported to relieve the bacterial burden in wounds. The ease of synthesizing these NPs and control over their shapes and sizes influence their properties, and it is of great importance in their antimicrobial activity. The antimicrobial activity and possible mechanisms of action of Ag and Cu NPs have been discussed in literature. Moreover, the effect of sizes and shapes on the toxicity of Ag and Cu NPs towards bacteria have been reported in various studies.

2.3.1.1 Silver nanoparticles

AgNPs are arguably the most applied and popular metal-based antimicrobial active agents based on previous studies. Scientific fields such as environmental and biomedicine have substantially benefitted from the antimicrobial activity of AgNPs. AgNPs have been used as disinfectants in water purification and bactericidal agents in wounds and infectious diseases [111]. The ability of AgNPs to unravel the dilemma associated with MDR bacteria is of paramount importance in human health. MDR bacteria such as *P. aeruginosa*, *E. coli*, *Salmonella enterica* (*S. enterica*) and *Proteus mirabilis* (*P. mirabilis*), which are respectively resistant to carbapenem, amoxicillin and ampicillin, were found to be susceptible to AgNPs [112-115]. A study by Cardozo *et al.* showed increased antimicrobial activity of AgNPs against the methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial strain [116]. Biasi-Garbin *et al.* showcased the antimicrobial effect of AgNPs against *Streptococcus agalactiae* (*S. agalactiae*), a bacterial strain that has shown considerable resistance against ampicillin and vancomycin antibiotics [117]. In a recent study by El-Sherbiny *et al.*, improved antimicrobial activity of biosynthesized AgNPs against various MDR bacteria and *Candida* isolated from a cancer patient was observed. Compared to antibiotics (i.e., amikacin, gentamycin, vancomycin and linezolid), the biosynthesized AgNPs showed a superior activity against bacteria and fungi [118]. Prepared from the supernatant of *Bacillus subtilis* (*B. subtilis*) strain, AgNPs were found to have antimicrobial effects against *E. coli*, MRSA, *P. aeruginosa* and *Acinetobacter baumannii* (*A. baumannii*) strains [119]. More recent studies showing the improved antimicrobial activity of AgNPs against MDR bacteria have been well documented in literature [120, 121].

The antimicrobial activity of AgNPs is affected by various parameters, including but not limited to their size and shape. Ajitha *et al.* showed a particle size-dependent antimicrobial activity of AgNPs against *E. coli* and *Pseudomonas species* (*Pseudomonas sp.*). An increase in antimicrobial activity with decreasing NPs sizes was observed against both strains. These observations were attributed to the increase in the size of the inhibition zones as the NPs were increasing in size. The improved antimicrobial activity of smaller NPs was attributed to their strong potential to interact more with bacteria due to their high surface area compared to bigger NPs [122]. Cheon and co-workers compared the antimicrobial activity of AgNPs with spherical, disk-like and triangular shapes. The spherical AgNPs showed an excellent antimicrobial activity followed by the discs and then the triangles against *E. coli*, *S. aureus* and *P. aeruginosa*. The large surface area of spherical AgNPs contributed to strong

antimicrobial activity compared to the disk-like and triangular [123]. Various mechanisms of action of AgNPs against bacteria have been reported; however, exact mechanisms are still a topic of debate. According to other reports, AgNPs damage the cell wall or cell membrane of bacteria leading to the leakage of organelles [124]. Some reports proposed that AgNPs rapture the bacterial wall, which leads to cell fragmentation [125, 126]. Li *et al.* suggested that AgNPs first interact with the bacteria's cell wall or cell membranes, which disturb their properties. This is followed by the penetration of AgNPs inside the bacterial cell, resulting in DNA damage. Thirdly, Ag NPs undergo dissolution resulting in the release of silver ions which can possibly interact with thiol-containing proteins inside the cell. Various studies also investigated the production of ROS by AgNPs and their ability to deplete antioxidants within the cells thereby inactivating the functionality of the bacteria [127].

2.3.1.2 Copper nanoparticles

CuNPs form part of the most effective and studied metal-based antimicrobial agents. Other than its great antimicrobial activity, Cu is relatively cheap compared to noble/ coinage metals such as Ag and Au, which makes the synthetic methods of Cu NPs cost effective and attractive for research [128]. Research has also shown the potential of CuNPs in fighting both gram-positive and gram-negative MDR bacterial strains [129]. Due to their excellent antimicrobial activity like AgNPs, CuNPs have also been applied in a wide range of biomedical applications such as drug delivery and wound healing [130, 131]. Researchers have suggested that CuNPs can be a very suitable and cheap alternative to AgNPs in applications requiring active antimicrobial agents. However, compared to AgNPs, the antimicrobial activity of CuNPs has been less studied and their mechanism of action toward bacteria is less understood.

Hassan and co-workers studied the antimicrobial activity of CuNPs against infectious microorganisms. An excellent antimicrobial effect towards fungi (*spergillus brasiliensis* ATCC.16404 and *Candida albican* ATCC.10231), gram-positive bacteria (*S. aureus* ATCC.6538, *B. subtilis* ATCC.6633 and *Bacillus dimenuta* (*B. dimenuta*) ATCC.19146) and gram-negative bacteria (*P. aeruginosa* ATCC.9022 and *E. coli* ATCC.8739) [132]. Kruk *et al.* investigated the antimicrobial activity of CuNPs against infectious MDR bacteria and fungi. The study evaluated the antibacterial and antifungal activity of the CuNPs against clinical and standard strains. The synthesized NPs exhibited an interesting antibacterial activity against various staphylococcus strains, including MRSA. The antifungal tests also

demonstrated an excellent activity against various clinical and standards strains of candida species [133]. In addition, a study by Usman *et. al.*, indicated the antimicrobial ability of CuNPs against various bacterial strains including *B. subtilis*, *P. aeruginosa* and MRSA [134]. According to Das *et. al.*, Cu NPs inhibited the growth of *S. aureus*, *B. subtilis*, and *E. coli*. Another study by Ramyadevi *et. al.*, showed a promising antimicrobial potential of CuNPs against *Micrococcus luteus* (*M. luteus*), *E. coli*, *S. aureus*, *Klebsiella pneumoniae* (*K. pneumoniae*) and *P. aeruginosa* [135]. Furthermore, a recent study by Asghar *et. al.* showed an antimicrobial activity of the green synthesized CuNPs using plant extracts. CuNPs strongly possessed an antimicrobial effect against gram-negative (*S. aureus* and *S. mutans*) and gram-negative (*E. coli*, *K. pneumoniae* and *S. typhi*) bacterial strains. Interestingly, these NPs were also able to inhibit the growth of the MDR *P. aeruginosa* strain [136]. The attractive antimicrobial activity of CuNPs has recently attracted research in various fields which is evident from increasing number of published studies [137-140].

Even though the mechanism of action of CuNPs is not very well understood as that of AgNPs, various reports have been published showing the possible mechanism of action of these NPs against microbes [87, 132]. CuNPs are believed to exert three universal mechanisms of bacterial killing than most metal NPs exhibit. Firstly, they interact and dissolve on the bacterial membrane, causing membrane rupture, which results in the release of lipopolysaccharides, proteins and other intercellular biomolecules. Secondly, they release ions from the NPs and produce ROS which increase oxidative stress and deplete antioxidants. Thirdly, is the uptake of ions derived during NPs dissolution and/or oxidation or in terms of CuNPs. At an appropriate concentration, the NPs and ions uptake deplete the intercellular ATP production. In other studies, it was suggested that Cu ions produced due to NPs oxidation and dissolution may interact with sulfur and phosphorus rich biomolecules such as proteins and DNA and disturb the normal functioning of the cells [88, 102, 141]. An interesting study conducted by Chatterjee *et. al.* revealed that CuNPs facilitate the depolarization of cell membranes which results in only bacterial cell elongation without cell division. In addition, the generation of ROS by NPs was found to cause bacterial cell death as a result of cellular lipid peroxidation, protein oxidation, and DNA degradation [142].

2.4 Polymers

2.4.1 Overview

A polymer is a large molecule built up by a repetition of chemical units known as monomers, bonding together. Some polymers are made up of a single type of monomer and are known as homopolymers [143]. For an example, polystyrene is made of repeating units of a single monomer called styrene [144]. Other examples of homopolymers include polyethylene, polyacrylonitrile, polypropylene and PDA. Copolymers are the ones that are derived from a mutual reaction of at least two monomers that are not identical [143]. Nylon is one of the common copolymers derived from the reaction of hexamethylenediamine and adipic acid. Other examples of copolymers include polyethylene terephthalate and proteins. In general, polymerization occurs through a successive series of reactions as the repeating units or monomers are linking together. Monomers link together to form dimers, and the dimers link with other monomers to form trimers and so on. Trimers, dimers and monomers can also react with each other until a bigger polymer chain is formed. The length of a polymer molecule is measured by a degree of polymerization (DP), which measures the number of repeating units in the polymer molecule. Small and low molecular weight polymer chains such as dimers, trimers, tetramers etc., are called oligomers. Polymer chain length or DP determines their properties, and oligomers possess undesirable chemical, thermal and mechanical properties since they have low DP [143, 145].

2.4.2 Classification of polymers

There are various ways through which polymers are classified. Polymers are commonly classified as either natural or synthetic. The other ways of classifying polymers are through preparation methods, their properties and structures, and mechanism of polymerization.

2.4.2.1 Natural vs Synthetic polymers

Natural polymers are polymers that occur naturally and are also known as biopolymers since they are of biological origin [146]. Since they are derived during life cycles of living organisms, natural polymers are biodegradable, which has attracted wide interest in applications' such as drug delivery and tissue engineering [147]. Examples of natural polymers include polysaccharides and proteins [145]. Starch, cellulose and chitin are the most abundant polysaccharides. Starch and cellulose make a large part of the bulk plants kingdom and are most abundant compared to chitin. The difference between cellulose and starch is their

structural conformation. The glucoside linkage is an α -acetal-hydrolysable to axial hydroxide in starch and β -acetal-hydrolysable to an equatorial hydroxide [148]. **Figure 2.3** shows the structural configuration of starch and cellulose. Chitin is a type of polysaccharide that is mostly derived from beetles and crabs' shells. This is the second most available polysaccharide after cellulose and starch. Compared to starch and cellulose, chitin is composed on an amide group which replaced one of the hydroxyl group presents in starch or cellulose. Chitin can be converted to CS, one of the most used polymers in biomedical applications including bone and skin tissue engineering (**Figure 2.4**) [149]. This polymer is obtained through replacing an amide group from chitin with a primary amine group as shown in **Figure 2.4** [150].

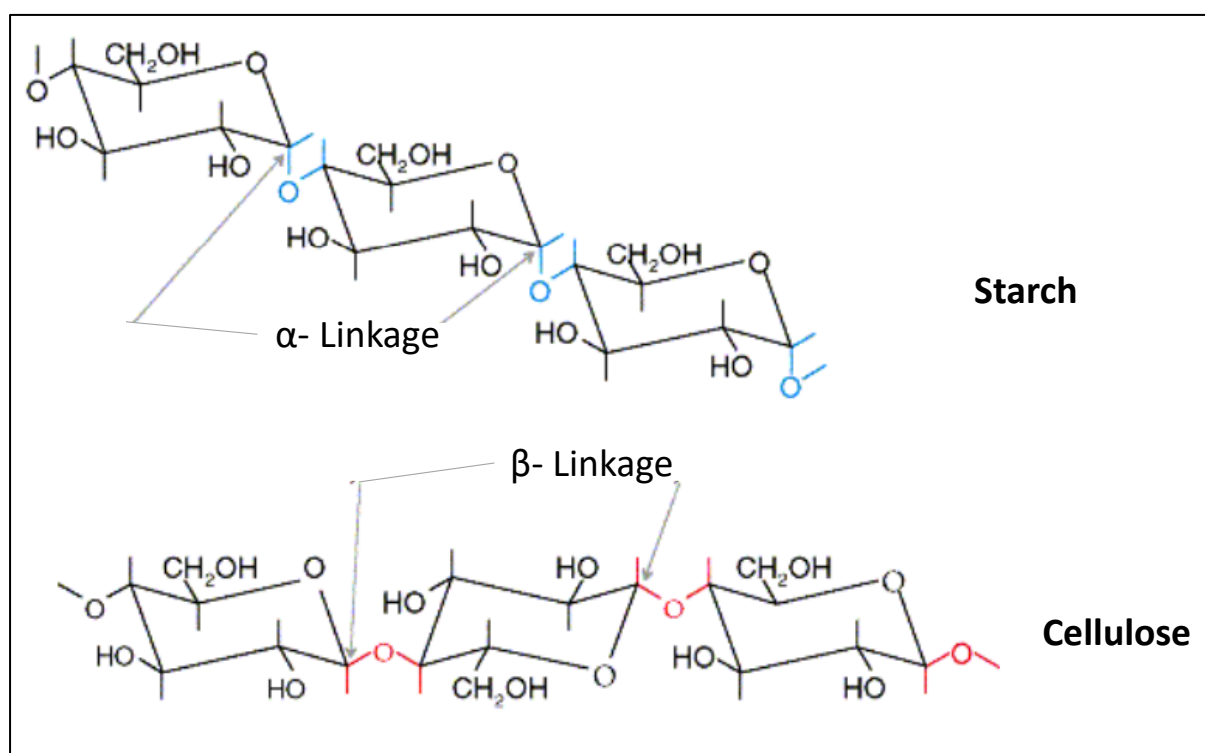


Figure 2.3: Structural conformation of starch and cellulose showing their distinctive glucoside linkage. Modified from [151].

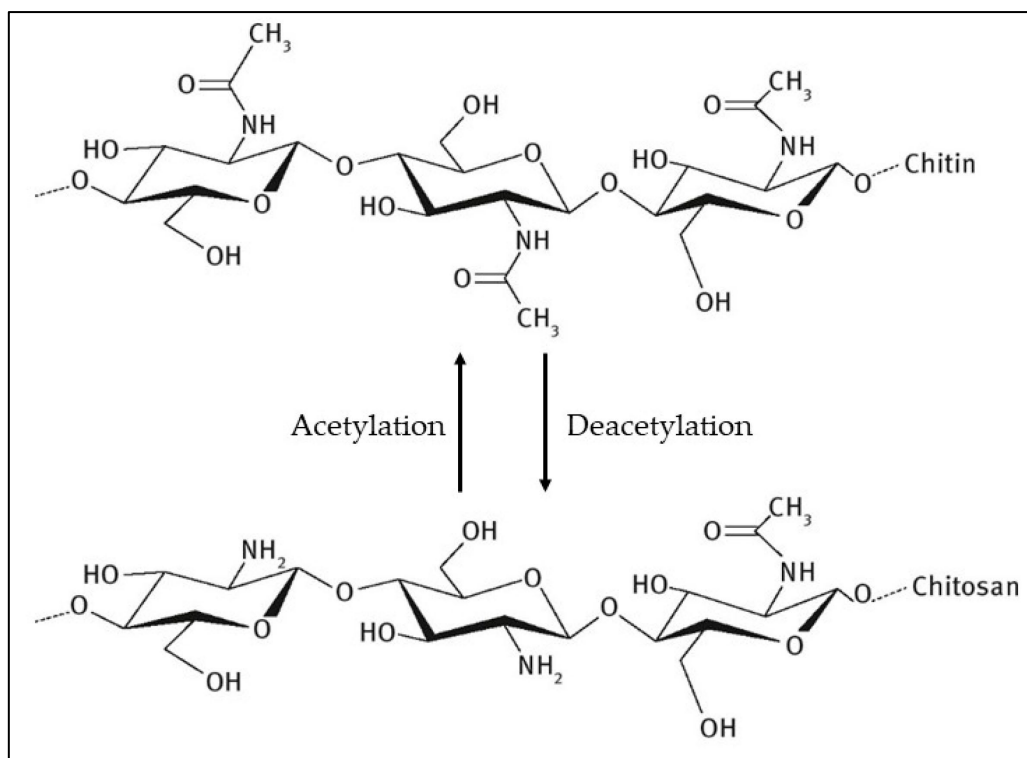


Figure 2.4: Chemical process showing the formation of chitosan from chitin and its respective reverse process. Taken as it is from [150].

Proteins on the other hands, are macromolecules also known as polyamides with various amino acids as a repeating unit. Proteins with molecular weights of less than 10 000 are called polypeptides. Proteins are characterized by their three structural levels namely, primary, secondary and tertiary structure. The primary structure is the sequence of amino acids in the polyamide chain, and the secondary structure refers to a specific spatial arrangements of a protein like α -helix and β -sheet, while tertiary structure refers to the overall shape of the protein [152]. Some examples of proteins are keratin, collagen and Gel. Keratin is a biopolymer that is mostly found in the epithelial cells of vertebrates. It known to be the most commercially available polymer, globally. It exists as α -keratin that is usually found in soft tissue like hair, sheep wools and skin, and as β -keratin that is usually found in hard tissue such as horns and claws. As a fundamental component of skin, this polymer has proven to play a crucial role in wound healing [153]. Collagen is the abundant protein in mammals which forms the major structural component of ECM; a network of macromolecules that forms a structurally stable composite that contributes to the mechanical properties of tissues. This polymer is usually found in human skin and bones. Collagen has been widely used in various applications including but not limited to wound healing. This is because of its

excellent biocompatibility and cell adhesion with the ability to promote cell proliferation and differentiation [154, 155]. Collagen can be transformed into Gel through thermal denaturation in the presence of dilute acid (**Figure 2.5**) [156]. Gel has similar physical, chemical and biological properties as collagen and it is relatively cheap. For these reasons, Gel is among the most used biopolymers in skin tissue regeneration [157, 158].

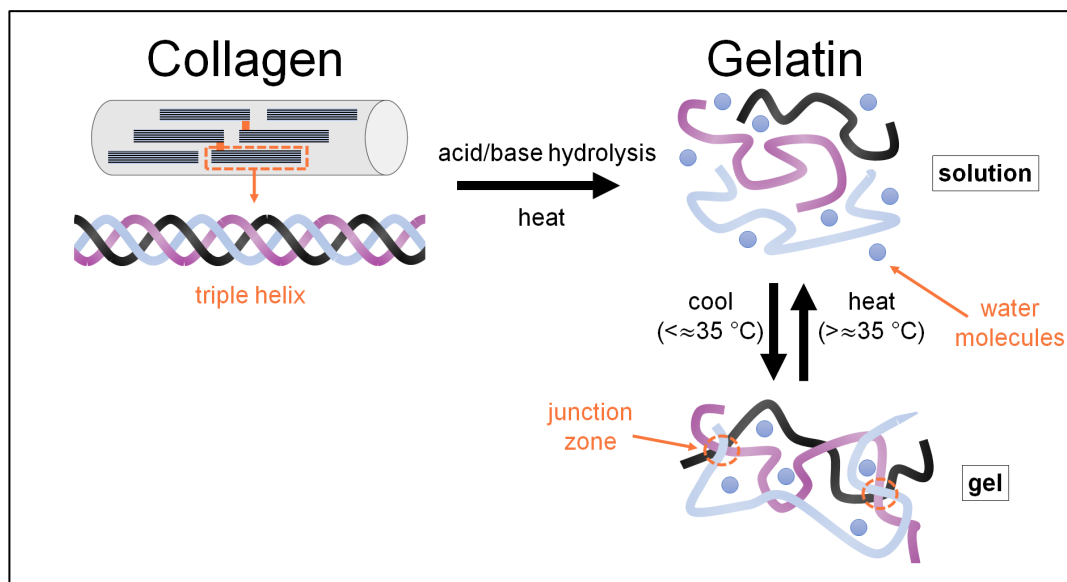


Figure 2.5: Formation of gelatine through collagen denaturation. Modified from [159].

Synthetic polymers are man-made polymers that are synthesized in laboratories. Most synthetic polymers are derived from petroleum oils with carbon-carbon bonds as their backbone and are referred to as organic polymers [160]. Synthetic polymers with backbones other than carbon-carbon bonds, e.g., polysiloxane with silicone-oxygen backbone, are known as inorganic polymers [145, 160]. Synthetic polymers are used in millions of everyday applications, however, in medicine and biomedical fields, biodegradable polymers are required. In skin tissue engineering, for instance, only polymers that can be able to integrate and be able to be degraded during biological processes can be used. Examples of synthetic polymers that are commonly used in pharmaceutical and biomedical fields include poly(ethylene glycol) (PEG), poly(propylene glycol), polyurethane (PU), poly(maleic anhydride) and polydopamine (PDA) [161-164]. **Table 2.1** summarizes common synthesis techniques used for preparation of synthetic polymers, while **Table 2.2** is comparing few characteristics of natural and synthetic polymers.

Table 2.1: Different types of polymerization synthesis techniques

Classification of polymerization synthesis techniques	
Synthesis technique	Description
Bulk polymerization	The bulk polymerization process consists of adding or converting a monomer to another. Only the monomer and the initiator or a catalyst are present in the reaction mixture. This process produces a highly purified product [165].
Solution polymerization	Solution polymerization involves using an inert solvent that has a boiling point that corresponds with the polymerization temperature. At the end of the reaction, the product is separated from the solvent [166].
Slurry polymerization	In a slurry polymerization, a catalyst is dispersed/dissolved in either a solvent in which the monomer is dissolved or in a liquid monomer. As the polymer is not soluble in the reaction medium, it precipitates as a slurry on the catalyst [167].
Suspension polymerization	The monomer disperses in water, forming small droplets of polymer. As a result, the polymer precipitates as small spheres. To avoid particle agglomeration and to produce uniform particles, protective colloids/stabilizing agents are used [168].
Emulsion polymerization	The emulsion polymerization process involves dispersing a monomer in water. By using an emulsifier (surfactant), very small droplets of monomer are formed (much smaller than when polymerizing in suspension). The polymer molecules disperse and form spherical particles as a result [169].
Gas-phase polymerization	In gas-phase polymerization, a monomer is used in the presence of a catalyst supported on a material such as alumina. In this way, polymers are formed on the catalytic sites [170].

Table 2.2: Comparison of the characteristics of polymers

Natural	Synthetic
Occur naturally (Properties controlled through naturally occurring reactions)	Man-made (Properties can be tailored by controlling reaction parameters)
Different repeating units	Identical repeating units
Biodegradable	Some are not biodegradable
Environmentally friendly	Some are not environmentally friendly
Limited recyclability	Some can be recycled

2.3 Wound healing properties of polymers and metal nanoparticles

2.3.1 Wound management

Suitable materials must be employed for efficient healing of wounds. These materials cover the wound so that they can help to prevent bacterial infections and exudates. In ancient times, the plants fibre, honeycomb and animal fats were utilized as wound dressing materials [171]. A wound dressing material is supposed to possess exceptional properties which promote the process of wound healing. The characteristic of the wound, type of the wound, healing time of the wound together with the physical and chemical properties of the materials used must be taken into consideration in order to design an effective and functional wound dressing. The main goal is to improve the rate of the healing process and to restore the skin tissues to their normal condition. Although various kinds of wound dressings are currently used in the management of wounds, infections under the wound dressing remain a formidable problem. Bacterial infections of wounds can significantly compromise the healing process and in most cases, prevent wound healing [172]. One of the most common methods for treating infections is through the use of antibiotics. Although antibiotics have been proven to be very helpful against wound infections, it has also been reported that their use can be associated with complications such as renal and liver toxicity and the emergence of drug-resistant bacteria [173]. Patients with chronic wounds are often treated with systemic antibiotics in an attempt to decrease the bacterial load present in the wound. However, it is known that despite the abundant blood supply present in the granulating tissue, the systemic administration often does not produce appreciable levels of antibiotics in granulating tissue. Furthermore, ischemic wounds with little granulation tissue also have poor penetration by systemically administered antibiotics. Therefore, the topical administration has proven to be an effective

approach. In the ideal case, the antibiotic is used in appropriate amounts at the site of the infection and for the duration of the infection [174-178].

To date, several biomaterials that regulate infections and accelerate the healing process in both acute and chronic wounds have been developed. Metal-based nanomaterials have been used in various applications within the biomedical field, ranging from diagnostics to therapeutics. This is because of their simple synthetic methods and their unusual size-dependent optical biological properties [87, 88, 123]. In addition, metal oxide nanomaterials have also been applied in wound healing, owing to their antimicrobial activity and their ability to inhibit bacterial biofilm formation. These oxide nanostructures offer another advantage since they readily generate ROS that can inhibit bacterial cell growth. Various metallic NPs such as zinc (Zn), gold (Au), titanium (Ti), copper (Cu) and silver (Ag) have shown excellent antimicrobial activity. Ag and Cu NPs have shown to possess the most effective antimicrobial activity compared to the other metallic NPs [87, 88, 103, 105-109]. These NPs have been exploited in various biological applications such as textile fabrics, dental materials, sunscreen lotions and wound treatment. In addition, the low toxicity of Ag and Cu NPs towards mammalian cells at monitored concentrations adds more advantage in biological applications including wound healing and drug delivery.

2.3.2 Wound healing properties of metal nanoparticles

2.3.2.1 Silver and copper in wound healing

The effectivity of wound dressings containing Ag and Cu NPs have been widely tested both *in vitro* and *in vivo*. Various research has proven that these dressings are effective and have both a fast and broad spectrum of antimicrobial activity against both gram-positive and gram-negative bacteria [33, 131]. However, the number of *in vivo* studies that have been conducted to date are still insufficient. Rigo *et al.*, demonstrated that the application of AgNPs-based wound dressing doesn't have any negative impact on the proliferation of keratinocytes and fibroblasts. This wound dressing also doesn't compromise cell viability and it is antimicrobial active, leading to complete skin restoration [179]. CuNPs on the other hand were reported to be crucial candidates in various wound healing processes. CuNPs-based wound dressings were found to enhance angiogenesis, increase the expression of integrin, up-regulate the activity of copper-dependent enzymes and polysaccharides important for matrix remodelling, cell proliferation, and re-epithelization, and increase the stabilization of fibrinogen and collagen [180]. The systematic administrations in severe burn trauma in children have

demonstrated the essential and benefits of using Cu in wound healing [181]. Since Cu is a crucial metal for perfect functioning of the metabolic system, its exposure to the wound in the form of NPs does not pose a health issue at controlled concentration [131].

Despite the fact that wound dressings containing these materials are safe and effective, previous studies have shown the possible health effects possessed by these materials. This includes the decrease in mitochondrial function and cellular shrinkage and irregular shape [182, 183]. A study by Carlson and co-workers showed that the production of ROS from AgNPs was the main issue for the health effects [184]. Hackenberg and co-workers also found that these ROS compromise the DNA structure of human mesenchyme stem cells. ROS production is dependent on the size, morphology and the concentration of NPs, and thus responsible for bacterial attack. Therefore, the ROS production can be controlled in order to reduce the toxicity of NPs [185].

2.3.3 Polymers in wound healing

Impressive efforts on improving and overcoming the shortfalls that are associated with conventional wound dressings have been made. Wound dressings in the form of scaffolds have proven to be promising for wound care treatment. Scaffolds are designed using either natural or synthetic polymeric material in 3D structures that can fit and integrate with a wound bed. They have proven to have the ability to accelerate the rate of wound healing since they can easily improve cell migration and proliferation, offer a conducive environment for wound healing, protect from bacterial contamination and most importantly they are flexible to fit in the wound [186, 187].

2.3.3.1 Skin tissue scaffolds from natural polymers

Natural polymers, also known as biopolymers, are derived from natural available sources such as plants, animals and microorganisms. These polymers offer several advantages compared to synthetic ones. This includes biocompatibility, biodegradability and biological activities. These polymers can easily mimic natural extracellular matrix (ECM) components and skin cellular background. The chemical structure of natural polymers is rich in reactive functional groups that can be linked with other chemicals or polymers to create versatile tissue scaffolds. The other advantage of natural polymers is that they produce harmless by-products when they degrade in the wound [187]. Studies Aderibigbe *et.al* and Kamoun *et. al.*, reported on the role of the alginate polymer in the wound healing process [188, 189].

Alginate is able to absorb wound exudates and maintain a moist environment. Most importantly, this polymer was found to be able to promote formation of granulation tissue and stimulate monocytes to produce cytokines. However, alginate lacks control over fibroblasts which can compromise the ECM and collagen in producing the structural framework of the skin [188]. CS on the other hand has shown the ability to improve the functions of fibroblasts, macrophages and inflammatory cells. It improves wound granulation, and its by-products take part in the makeup of ECM. CS has been reported to be antimicrobial active which is one of the main characteristics required from a wound healing scaffold or any other type of wound dressing [53, 59, 190, 191]. CS, however, is less stable and can degrade faster before the wound has healed, which is a major drawback [191]. Collagen and its derivative, Gel, have also shown great potential in skin tissue regeneration. Collagen is the major fibrous structural protein in the ECM that provides tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development [192]. Gel is a denatured form of collagen with excellent biodegradability and is more cost effective. This polymer has the ability to facilitate cell adhesion and proliferation [50]. As much as they can offer countless positive characteristics to be applied in wound healing as tissue scaffolds, natural polymers have an uncontrolled rate of degradation. This can compromise their wound healing properties since they can degrade before the complete healing process is done. Therefore, to improve and control their mechanical stability and degradability, these polymers can be functionalized with other groups such as synthetic polymers. This will not only improve their stability and degradability but incorporating natural polymers with synthetic ones can also improve their wound healing properties [192].

2.3.3.2 Skin tissue scaffolds from synthetic polymers

Synthetic polymers are made in laboratories. As a result of recent advances in synthetic and processing methods, and the availability of monomeric compounds, new synthetic biocompatible and biodegradable polymers have recently been developed [145, 192]. These polymers can be synthesized in a controlled manner to achieve the required physical and chemical properties for a certain application [145]. Some of the problems associated with natural polymers can easily be overcome using synthetic polymers or a combination of both synthetic and natural polymers. Unlike with natural polymers, the stability of synthetic polymers can be controlled, thereby controlling their degradability. Synthetic polymers have been reported to degrade through chemical hydrolysis and are not very sensitive to various

enzymatic processes. As a result, a number of wound dressings designed from synthetic polymers have been reported, especially for chronic wound healing [143, 145, 192, 193]. Polyvinyl alcohol (PVA) is one of the polymers that have been widely used in wound healing due to its hydrophilicity, non-toxicity and biocompatibility properties. PVA is bioadhesive and has oxygen permeability properties which makes it a good candidate for wound healing [194]. PEG is another synthetic polymer with properties similar to that of PVA as far as wound healing is concerned. PEG hydrogels have shown to be advantageous for diabetic wound healing since they enhance cell proliferation, induce collagen deposition and reduce formation of scars [195]. Water absorption and oxygen permeability properties of PVP have made it one of the polymers suitable for wound healing [196]. PU, PGA, PLA, and PLGA fall amongst popular synthetic polymers with great potential in wound healing applications [197-199]. These polymers can provide high strength and appropriate degradation and biocompatibility for skin tissue engineering. Most importantly, these polymers can promote a faster healing process and reduced inflammatory infiltrate [194, 196, 198]. However, some of the synthetic polymers have poor mechanical properties (such as strength and flexibility) and compromised hydrophilicity which can present some complications in wound healing [200]. Blending synthetic polymers with some natural polymers (such as chitosan, dextran, hyaluronic acid) and/or other synthetic polymers has proven to improve their wound healing properties [201, 202]. Polymers are usually blended using cross-linking methods to create improved tissue scaffolds with optimal wound healing environment. Cross-linking methods combine polymers using cross-linkers such as genipin, oxidized dextran or glutaraldehyde [203]. Mndlovu *et al.* developed a tissue scaffold from alginate cross-linked with chitosan with fluid absorptive properties [204]. Cross-linking these two polymers has shown to have improved porosity, degradation and water uptake of the final scaffold. A collagen/chitosan scaffold loaded with silver nanoparticles designed by You *et al.* showed potential wound healing properties *in vitro* and *ex vivo* [205]. In *in vitro* studies, this scaffold showed acceleration of fibroblasts with an increase in the expression of α -smooth muscle actin (α -SMA). An increased level of pro-inflammatory and scar-related factors were also observed, while microphage activating markers were up-regulated. It is important to note that the prepared scaffold was antimicrobial active as a result of AgNPs loading. Tissue scaffolds loaded with antimicrobial active agents have attracted research interest in the biomedical field.

2.4 Incorporation of metal NPs in polymers for wound healing

Chronic wounds such as venous and diabetic ulcers among others affect billions of people on a yearly basis worldwide. These wounds usually lead to mutation, amputation, or even mortality. This is due to the fact that most chronic wounds fail to heal permanently and on time. Therefore, advanced and novel wound treatment methods that offer infection control, sharp debridement, and weight bearing restriction are needed. Recently, metal/polymer nanocomposites have attracted considerable attention in the field of science due to their outstanding physico-chemical properties [2, 168, 206, 207]. Antimicrobial and/or biodegradable polymer-metallic nanocomposites have proved to possess a broad spectrum of antimicrobial properties and prospective applications in public health care and biomedical field [37, 208, 209]. Various studies have reported on the synthesis of metal/polymer nanocomposites with improved wound healing properties [210, 211]. For instance, incorporation of biodegradable polymers such as PDA with metallic nanoparticles offers higher mechanical strength and electrical conductivity to the scaffolds [212-214]. PDA can be easily obtained from the self-polymerization process of dopamine under basic conditions. This biodegradable polymer has been applied in various interfaces of different fields including surface modification and biological applications. PDA offers various advantages such as good thermal stability, easily controllable size as well as a uniform spherical shape [215, 216]. Moreover, the presence of catechol and N-H groups provide PDA with an active surface for absorbing and reducing metal ions. This polymer has been applied in the field of biomaterials since they have good biocompatibility and non-toxic properties. Luo and co-workers have presented the antibacterial properties of PDA loaded with AgNPs [217]. These nanocomposites were found to be more antimicrobial active compared to AgNPs activity. Wu *et al.* also studied the antimicrobial activity of AgNPs incorporated with PDA towards gram-positive and gram-negative bacteria [218].

2.5 Remarks

Health care professionals and institutions face a profound problem because of the increasing number of patients with chronic diseases and infections. Most of these patients develop complicated chronic wounds at some stage. Chronic wounds are difficult to treat since they are prone to bacterial infections and don't follow the right stages of the healing process. New and advanced wound dressings/scaffolds with advanced properties suitable to keep an optimal wound healing environment need to be developed. Wound care affects everyone, from the school yard to the nursing home, a wide variety of wounds occur. Regardless of the origin of a wound, healing a wound quickly, preventing infection and reducing scarring is

critical. Our project basically focuses on developing a new advanced wound dressing with a novel technology to tackle bacterial infections and also accelerate the wound healing process.

2.6 References

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Chapter 3

Dopamine assisted synthesis of antimicrobial active silver nanoparticles and their cytotoxicity towards fibroblasts cells

3.1 Background

The outbreak of infectious diseases caused by pathogenic bacteria and the development of new antibiotic-resistant bacterial strains have posed a risk to human health. New and effective antimicrobial materials need to be developed to tackle the health hazards caused by pathogenic bacteria [1-4]. The past few decades have witnessed a plethora of various synthetic antimicrobial active materials, thanks to nanoscience and nanotechnology [3] [5, 6]. Synthesis of nano-based materials introduces characteristics and properties that cannot be imagined in bulk materials. Silver (Ag), in the form of silver sulphadiazine, silver nitrate, or silver-polymer composites has been widely studied and believed to be the most effective metallic antimicrobial agent [7-9]. In its nanoscale, Ag has caught scientists' and researchers' attention in various fields including biochemistry, biology and microbiology as antimicrobial agents [1, 6] [10, 11].

The antimicrobial activity of silver nanoparticles (AgNPs) has been widely investigated in the past years. AgNPs possess a broad inhibitory spectrum against microorganisms such as viruses, fungi and bacteria. The use of AgNPs as antimicrobial agents can be a potential solution to improve antibiotic activity. This is because most pathogens develop resistance against various antibiotics with time [1]. In this case, *Salmonella typhi* (*S. typhi*) has developed resistance against chloramphenicol, ampicillin, quinolone and trimethoprim [12]. Methicillin and fluconazole have lost their activity towards *S. aureus* and *Candida albicans* (*C. albicans*) [13]. Moreover, various antibiotics such as ampicillin, kanamycin, sulfisoxazole, streptomycin, tetracycline and ticarcillin have also lost their activity towards *E. coli* [14]. As a proof of concept, the antimicrobial effect of AgNPs with improved activity against both gram- (positive and negative) bacterial strains has been reported in various studies [15-18]. As a result, AgNPs have also been used in many other applications including but not limited to wound healing [19, 20]. In wound healing, AgNPs are incorporated in wound dressings or added in ointments. This incorporation has been

reported to help in preventing bacterial infections and reducing pain [21]. However, the cytotoxicity of AgNPs towards healthy cells can be their limiting factor in various biomedical and related fields. Hence, controlling the concentration, sizes and other parameters of AgNPs is very crucial.

The cytotoxicity of AgNPs is yet to be addressed in full, with consistent results. Various studies have investigated the toxicity of AgNPs from different angles. This included but was not limited to their sizes and concentration [22]. It was found that AgNPs with diameters of 10 nm negatively affected the cell viability of human lung cells in comparison to those of 40, 50 and 75 nm [22]. In another study, reduced cell viability in human mesenchymal stem cells due to AgNPs with a concentration of 10 µg/mL and particle diameter of < 50 nm was reported [23]. Contrary to the above reports, the non-toxic nature of AgNPs at a concentration of about 100 µg/mL with the particle size range of 10 – 20 nm was observed [24]. Although AgNPs possess several advantages that render them good materials for various applications, they are less stable compared to other coinage metallic nanomaterials and tend to agglomerate with time. Agglomerated AgNPs are known to be more toxic against healthy cells compared to well-suspended AgNPs [25]. Therefore, the synthesis of stable AgNPs is of great importance and can yield more desirable results in their applications. To address the stability challenges posed by AgNPs, researchers have introduced various stabilizing/capping agents to minimize agglomeration.

Various methods for synthesizing stable AgNPs have been reported in literature [26, 27]. The use of organic compounds from plant extracts such as yeba mate, and polymers such as polyvinylpyrrolidone (PVP) as capping and reducing agents in the synthesis of AgNPs have been found to play a significant role in stabilizing the nanoparticles, hence resulting in no signs of agglomeration [28-33]. Dopamine (DA) is an organic compound that functions as both a hormone and neurotransmitter in the body and brain, thus, it is biocompatible and less toxic compared to other capping agents. It is known to be rich in catechol and N-H functional groups which can reduce metal ions [34]. This study, therefore, reports on the controlled synthesis of DA-capped AgNPs (AgNPs-DA) of various sizes and further investigates their antimicrobial activity and cytotoxicity. To the best of our knowledge, the colloidal synthesis of AgNPs with controlled sizes using DA as a reducing agent has never been reported before. The antimicrobial activity of AgNPs-DA was investigated against *E. coli* and *S. aureus* bacterial strains. AgNPs-DA showed excellent antimicrobial activity against both bacterial strains at relatively low concentrations. The cytotoxicity of the nanoparticles towards BHK-

21 cells was also investigated. It was noticed that AgNPs-DA did not have a significant toxic effect on the cells with a viability of over 70% at the concentration of 27 $\mu\text{g/mL}$.

3.2 Experimental section

3.2.1 Materials

Silver nitrate (AgNO_3 , <99.0%), dopamine hydrochloride, Luria Bertani Broth (LB), Tryptic Soy Agar (TSA), dimethylformamide (DMF, 99.9%), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were all purchased from Sigma Aldrich and used as received. All glassware was washed with aqua-regia and rinsed with distilled water.

3.2.2 Preparation of AgNPs

To synthesize different sizes of AgNPs-DA, various amounts of silver nitrate (AgNO_3) were transferred in 100 mL of distilled water to give different concentrations (0.05, 0.10, 0.5 and 1 mM). The solutions were then boiled followed by the addition of dopamine hydrochloride (1 mL, 1 mM) for each AgNO_3 concentration. The reactions were left to take place for 5 min while stirring. A stable pale-yellow colour confirmed the formation of colloidal AgNPs-DA. The products were cooled and washed with distilled water before characterization.

3.2.3 Cell viability studies

The toxicity of AgNPs against mammalian cells was determined using the Baby Hamster Kidney-21 (BHK-21) cell line and cell viability was assessed using the MTS cell proliferation assays. BHK-21 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin antibiotic solution. The cells were grown in a 37 °C incubator with a humidified atmosphere of 5% CO_2 . For the assay, the cells were seeded at a density of 1×10^6 cells/mL and a final volume of 100 μL /well in 96-well tissue culture plates. The cells were incubated at 37 °C under 5% CO_2 for 48 h to allow for stabilization. Two-fold serial dilutions of AgNPs-DA in complete culture media were then introduced, with final concentrations on the plates ranging between 1.7 – 215 $\mu\text{g/mL}$. The plates were incubated under standard culture conditions for 48 h, after which the viability of the cells was determined using the MTS dye. The MTS solution, at a volume of 20 μL /well, was added and the plates were incubated at 37 °C for an additional 4 h. The optical density (OD) was then determined at 490 nm using a plate reader. The untreated BHK-21 cells (cells without AgNPs-DA) were used as negative control, while cells treated with Auranofin being used as a positive control. The percentage of viable cells (%Viability) was calculated using the following equation:

$$\% \text{Viability} = \frac{\text{Absorbance of Sample} - \text{Absorbance of Medium}}{\text{Absorbance of Control} - \text{Absorbance of Medium}} \times 100$$

3.2.4 Antimicrobial activity tests

3.2.4.1 Well diffusion method

The antimicrobial activity of the materials was tested using the conventional well-diffusion method against *E. coli* and *S. aureus*. The bacterial strains were each inoculated into LB Broth and cultured in a shaking incubator at 37 °C and 250 rpm overnight. The bacterial cultures were then spread-plated onto TSA plates. Wells were made on the plates using a cork borer and 100 µL of AgNPs-DA solution was added to the wells. After incubation at 37 °C for 18 - 24 h, the zones of inhibition were measured using a ruler. To ensure the reproducibility and obtaining the scientific sound results, the experiment was conducted in triplicate.

3.2.4.2 Minimum inhibitory concentration (MIC) and bacteriostatic studies

The MIC test was conducted using the standard broth dilution method. Two-fold dilutions of AgNPs-DA, with final concentrations ranging between 0.5 and 30 µg/mL, were prepared in 2 mL broth solutions inoculated with *E. coli* and *S. aureus*. The bacterial cultures were incubated at 37 °C for 24 h while shaking at 250 rpm. The concentration of bacterial cultures was adjusted to 1x10⁶ CFU/mL using the McFarland method. For bacteriostatic studies, the optical density of the solutions was measured at 600 nm. The study was conducted over 14 days. To ensure the reproducibility and obtaining the scientific sound results, the experiment was conducted in triplicate.

3.2.5 Characterization techniques

All excess impurities from colloidal solutions were removed using a universal 320R centrifuge at a maximum speed of 15000 rpm. The absorption spectra were acquired using a Varian Cary Eclipse (Cary 50) UV-vis spectrophotometer. Transmission electron microscopy (TEM) images were obtained from FEI Tecnai-T12 microscopy at an electron acceleration voltage of 120 kV and beam spot size of 10-100 nm. Powder X-ray diffraction (PXRD) analysis was performed on a Bruker D2 Phaser with Cu Kα. The Zeta potentials of the nanoparticles were obtained from a Malvern Zetasizer nano series using folded capillary cell (DTS1070).

3.3 Results and discussion

3.3.1 AgNPs-DA characterization

In this study, DA was simultaneously used as the reducing and capping agent. The oxidation of the catechol groups in DA was responsible for the reduction of silver (Ag) ions. The resulting dopamine quinone compounds adsorb on the surface of AgNPs-DA accounting for the size control and stabilization of the nanoparticles. **Figure 3.1** shows the TEM micrographs of AgNPs-DA and their particle size distribution are reported in **Figure S3.1**. The as-synthesized AgNPs-DA were spherical-like in shape, with average diameters of 16 nm, 24 nm, 48 nm and 74 nm as shown in Figure1 (A, B, C and D), respectively. The wide range of nanoparticles diameter observed from their size distribution signify polydispersity of the as-synthesized nanoparticles. The sizes of the nanoparticles were observed to increase with an increase in the concentration of AgNO₃. At a high concentration of AgNO₃, more Ag ions were reduced and nucleated, resulting in the fast growth of AgNPs through Ostwald ripening [35].

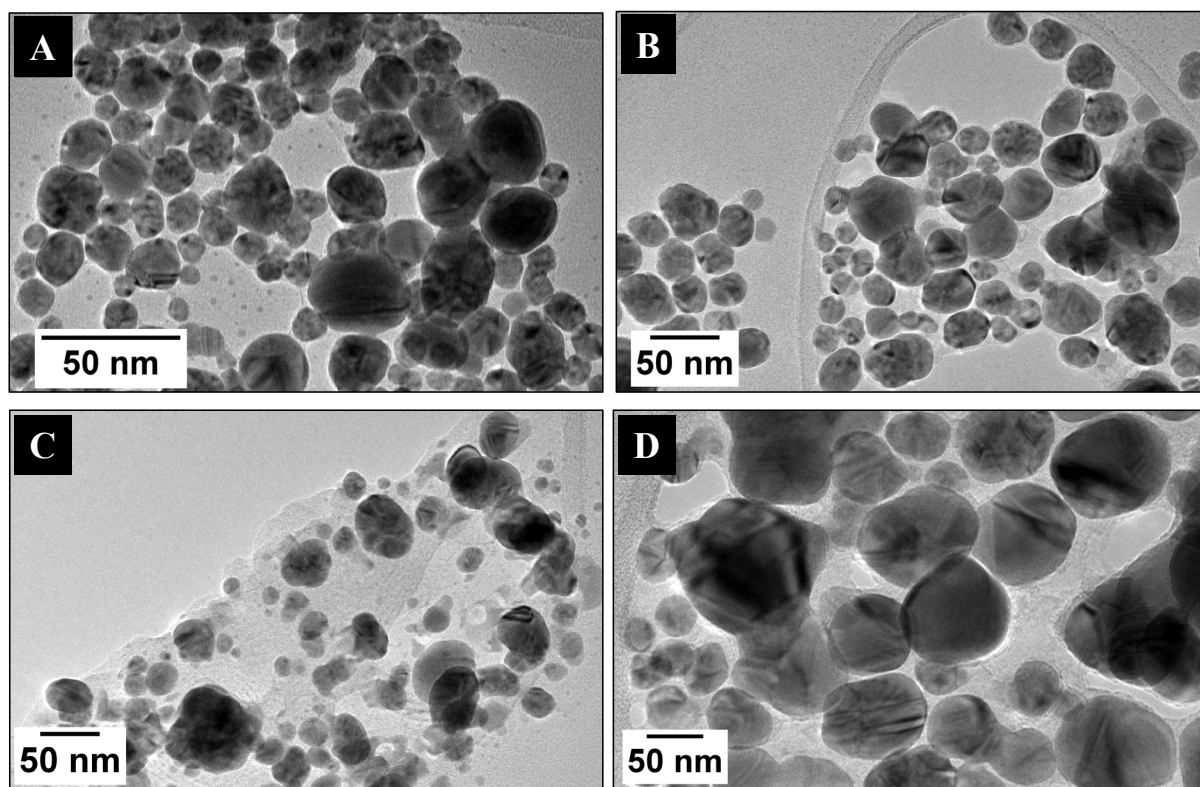


Figure 3.1: TEM images (A = 16 nm $\pm$ 6 nm, B = 24 nm $\pm$ 2.1 nm, C = 48 nm $\pm$ 3.2 nm and D = 74 nm $\pm$ 2.8 nm) of DA capped AgNPs synthesized at different AgNO₃ concentrations.

To study the optical properties of the synthesized AgNPs-DA, UV-vis absorption spectrometry was used. The UV-vis absorption spectra of AgNPs-DA synthesized at different AgNO₃ concentrations are shown in **Figure 3.2**. The characteristic surface plasmon resonance (SPR) bands were observed with maxima peaks appearing at 416, 432, 418 and 435 nm for AgNPs-DA with an average diameter of 16, 24, 48 and 74 nm, respectively. The presence of the SPR bands is an indication of the successful formation of AgNPs-DA. A clear and significant shift to higher wavelengths was observed as the size of AgNPs increased.

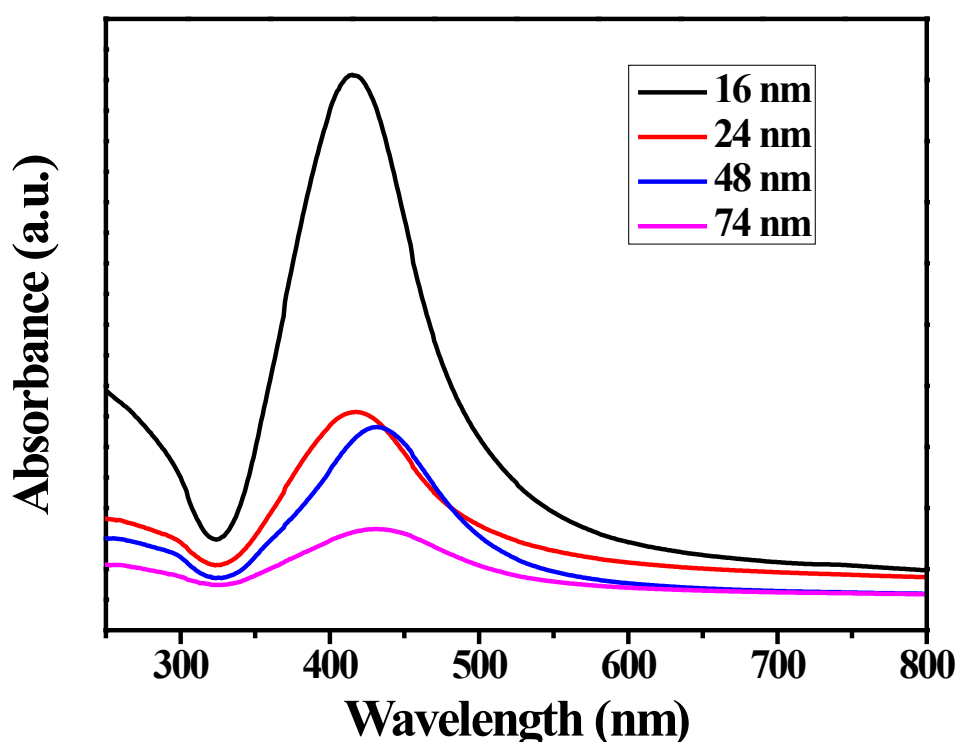


Figure 3.2: UV-vis absorption spectra of AgNPs-DA of various sizes.

The zeta potentials of the nanoparticles were determined using a Zetasizer to evaluate their surface charge and ionic strength. The ionic strength can be used to determine their stability. Generally, the colloidal nanoparticles with zeta potential above +60 mV and below -60 mV are considered highly stable, while the ones above +20 mV and below -20 mV are fairly stable [28]. As shown in **Figure S3.2**, the zeta potentials of the AgNPs-DA in this study were -20.3, -23.4, -25.6 and -26.5 mV for AgNPs-DA with the average diameters of 16, 24, 48 and 74 nm, respectively. This indicates that the nanoparticles are fairly stable. The negative zeta potentials suggest that the surface of the AgNPs-DA is negatively charged. This can be attributed to the adsorption of dopamine-semi-o-quinone anions on the surface of nanoparticles because of DA oxidation. It is important to note that the zeta potential of the

nanoparticles increased with nanoparticles diameter. This means that nanoparticles with a small diameter (16 nm) are less stable compared to bigger particles. This data is per the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory, which determines the total potential energy by summing the contributions from the van der Waals attraction and the electric double layer repulsion. According to the DLVO predictions, the smaller particles are more prone to agglomeration due to the decrease of interaction potential with particle size [36]. The other explanation for the low stability of AgNPs-DA with an average diameter of 16 nm can be the high surface energy posed by smaller nanoparticles in colloids. Due to the high surface energy of small nanoparticles, the activation energy gets lowered as a result of decreasing surface charge which can lead to particles agglomeration [37]. In addition, the inherent stability of small nanoparticles gets compromised due to their high specific surface area and a large fraction of surface atoms which promotes more reactive sites [38].

Chemical structures of DA and AgNPs-DA were studied using FT-IR spectroscopy as shown in **Figure S3.2**. The FT-IR spectra show well-defined characteristic functional groups of DA. The broad vibrational band around 3250 cm^{-1} shows the overlapping stretching modes of O-H and N-H functional groups. The sharp peaks around 1700 cm^{-1} and 1250 cm^{-1} are attributed to C=O and C-O vibrations respectively, while the peak at 1500 cm^{-1} represents the vibrational modes of C=N and C=C. The AgNPs-DA spectra displayed almost all vibrational bands that are present in the pristine DA spectrum. These spectra have aided in elucidating the interaction between Ag and DA. The bands of AgNPs-DA are slightly broadened and shifted to the higher wavenumber compared to those of DA. The broadening and the shifting of the bands could be the indication of the interaction between Ag and DA through hydrogen bonding which could promote the stability of AgNPs.

PXRD was used to investigate the crystallinity and structural phase of AgNPs-DA. The diffraction patterns of AgNPs-DA are shown in **Figure 3.3**. The diffraction patterns show diffraction peaks at 38.09° , 44.35° , 64.48° , 77.39° and 81.72° 2θ values, corresponding to (111), (200), (220), (311) and (222) lattice planes of face centred cubic (FCC) crystal structure of metallic Ag, respectively. The presence of the above peaks confirms the successful synthesis of AgNPs. However, as the concentration of AgNO_3 was increased, some peaks that were not due to metallic Ag (denoted by *) were observed. The presence of those unassigned peaks was a result of silver chloride (AgCl). When dopamine hydrochloride

oxidizes, most of the Ag ions get reduced into their zerovalent state. The excess Ag ions interact with the chlorine ions to form crystalline AgCl.

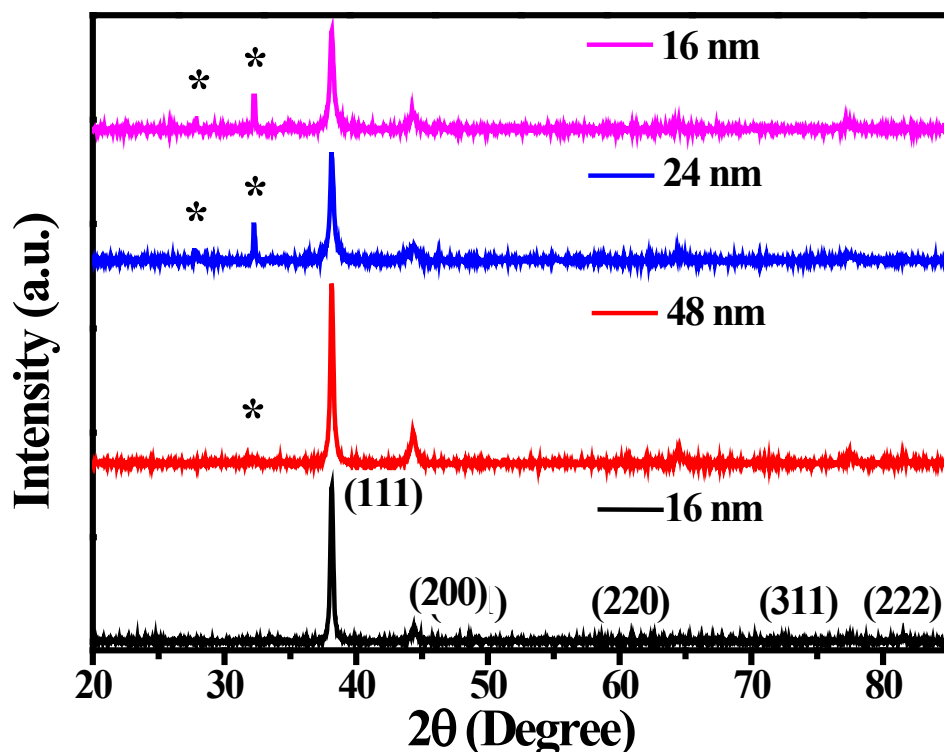


Figure 3.3: The PXRD patterns of AgNPs-DA showing Ag (PDF: 01-071-4612) and AgCl (* PDF: 00-031-1238) with the average diameters of 16 nm, 24 nm, 48 nm and 74 nm.

3.3.2 Toxicity studies of AgNPs-DA

The MTS assay was used to evaluate the cytotoxicity of AgNPs-DA. This was done by assessing the viability of BHK-21 cells following treatment with AgNPs-DA at various concentrations for a period of 72 h. The cells treated with only DMEM were used as a control study. A cell viability of approximately 100% was observed for the untreated cells, indicating that the cells were not affected throughout the study. The concentration of AgNPs-DA played a significant role in their toxicity as compared to their sizes. This is illustrated in **Figure 3.4**, which shows the viability of BHK-21 cells plotted against the concentration of AgNPs-DA with different sizes. A decrease in cell viability was observed as the concentration of AgNPs-DA was increased. Initially, the BHK-21 cells showed good viability at a concentration range of 2 to 27 $\mu\text{g/mL}$. At this range, the cell viability was over 70% for all the different sizes. A drastic decrease (to below 40%) of cell viability was observed when the concentration of AgNPs-DA was increased to 54 $\mu\text{g/mL}$. A further decrease (to below 30%) was also observed as the concentration was increased to 108 $\mu\text{g/mL}$. This simply signifies that the as-synthesized AgNPs-DA become toxic when the concentration exceeds 50 $\mu\text{g/mL}$, regardless of the size. Similar findings were previously reported, where AgNPs showed the

concentration-dependent cell viability in renal epithelial (A498) and hepatic (Hep G2) cell lines [39]. No clear trend was observed concerning the effect of AgNPs-DA sizes as the cell viability fluctuates. However, a slight trend was observed at high concentrations of AgNPs-DA. The cell viability was found to decrease with a decrease in the size of AgNPs-DA at the concentration of 54 and 108 $\mu\text{g/mL}$. In a separate study, it was also observed that AgNPs with small sizes pose a more negative impact on cell viability than bigger particles [40]. This is in concise agreement with our observations even though the trend was only clear at high concentrations.

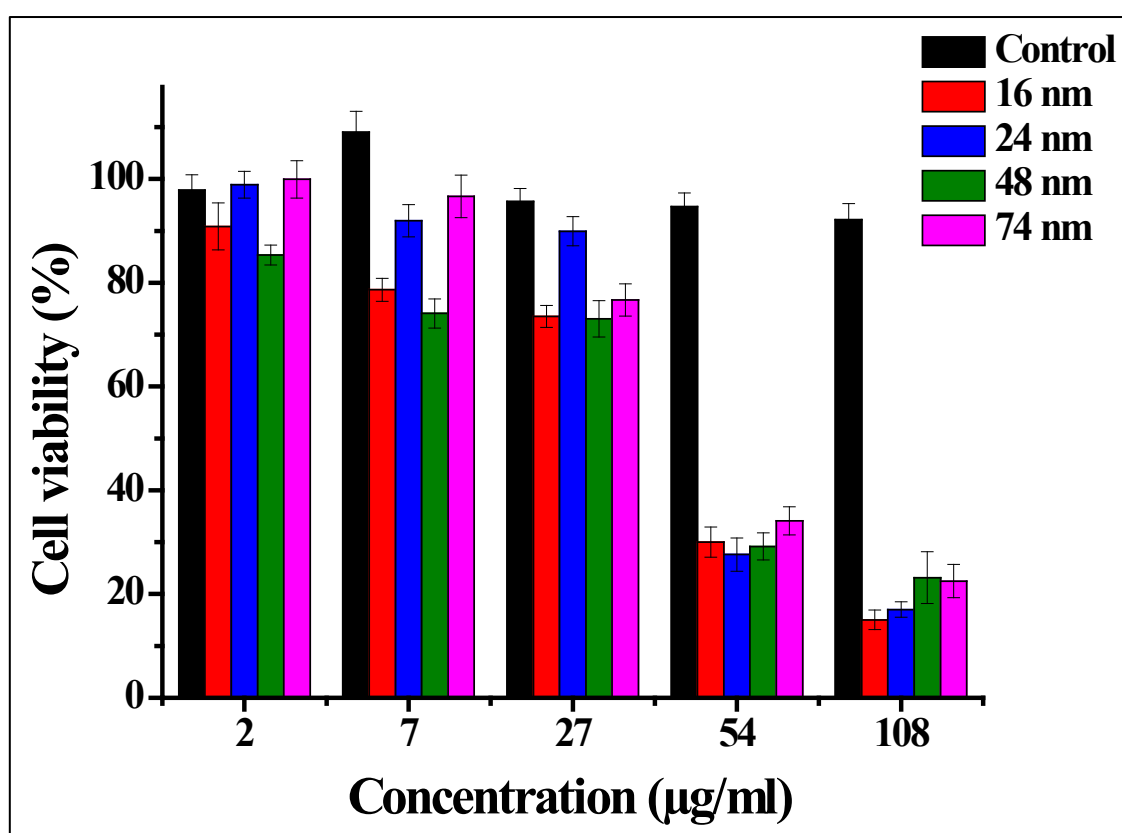


Figure 3.4: Cell viability of BHK-21 cells exposed to AgNPs-DA of different sizes at different concentrations.

Figure 3.5 shows representative sigmoid curves of BHK-21 cells treated with 16 nm, 24 nm, 48 nm and 74 nm AgNPs-DA. These were used to determine the 50% cytotoxic concentration (CC_{50}) values of AgNPs-DA. The CC_{50} value is defined as the concentration of AgNPs-DA that has reduced the cell viability by 50%. As shown in **Table 3.1**, the CC_{50} values for 16, 24, 48 and 74 nm AgNPs-DA were found to be 22.4, 30.9, 18.9 and 21.4 $\mu\text{g/mL}$, respectively. The CC_{50} values obtained show that AgNPs-DA did not exert significant toxicity on the

BHK-21 cells. There was no clear trend observed between CC₅₀ values and the sizes of nanoparticles, as was the case with the cell viability in **Figure 3.4**.

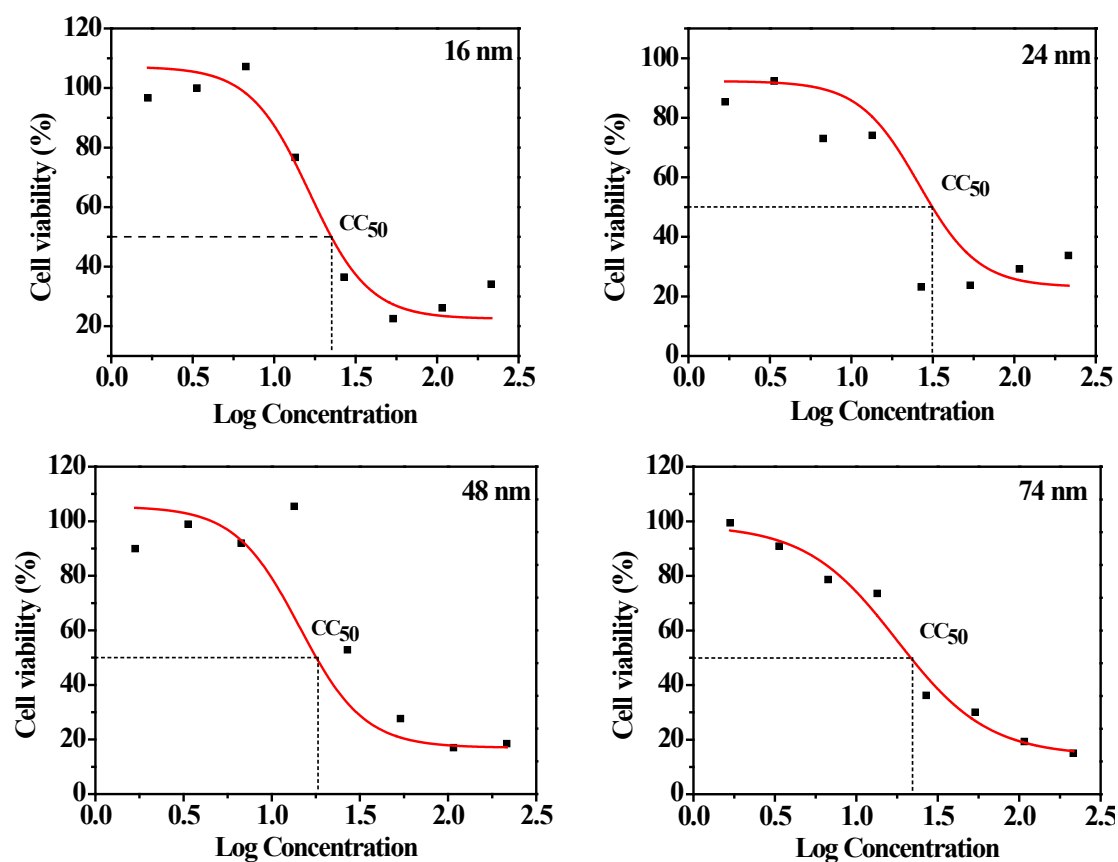


Figure 3.5: The sigmoid curves of cell viability acquired by incubating BHK-21 cells with 16 nm, 24 nm, 48 nm and 74 nm AgNPs-DA at different concentrations.

3.3.3 Antimicrobial activity of AgNPs-DA

The antimicrobial activity of AgNPs-DA was determined by the standard well-diffusion method against *E. coli* and *S. aureus* bacteria. **Figure 3.6** shows the photographed images of the agar plates with clear zones of inhibition around the wells. The zones of inhibition indicate the inhibition of bacterial growth by the nanoparticles. The effect of nanoparticles size on the inhibitory zones is also shown. The measured zones of inhibitions ranged from 9 mm to 17 mm and are summarized in **Table 3.1**. The as-synthesized AgNPs-DA, with varying sizes, exhibited antibacterial activity against both *E. coli* and *S. aureus*. The sizes of the zones of inhibition were observed to increase as AgNPs-DA average diameters decreased, indicating that smaller sized AgNPs-DA are more potent compared to bigger ones. This is attributed to the fact that nanoparticles with smaller sizes have a high surface area to volume ratio which makes it easier for them to penetrate the bacterial cell wall and interact with vital

organelles within the cell. In addition, the ability of AgNPs to release Ag ions (Ag^+) when in contact with the bacteria plays an important role in their toxic effect on bacteria cells. The release of Ag^+ ions facilitate the interaction of Ag with vital cell organelles which are lethal to bacteria. The comparison of the antimicrobial activity of the zerovalent Ag (Ag^0) and Ag^+ has been conducted in literature [41-43]. The inhibitory effect of the nanoparticles was stronger against *E. coli* than *S. aureus*. This was indicated by the larger zones of inhibition in *E. coli* compared to *S. aureus*. This may be due to the different cell membranes of the gram-positive and gram-negative bacterium. Gram-negative bacteria have a thinner peptidoglycan layer compared to gram-positive bacteria, making it easier for nanoparticles to penetrate gram-negative bacterial strains.

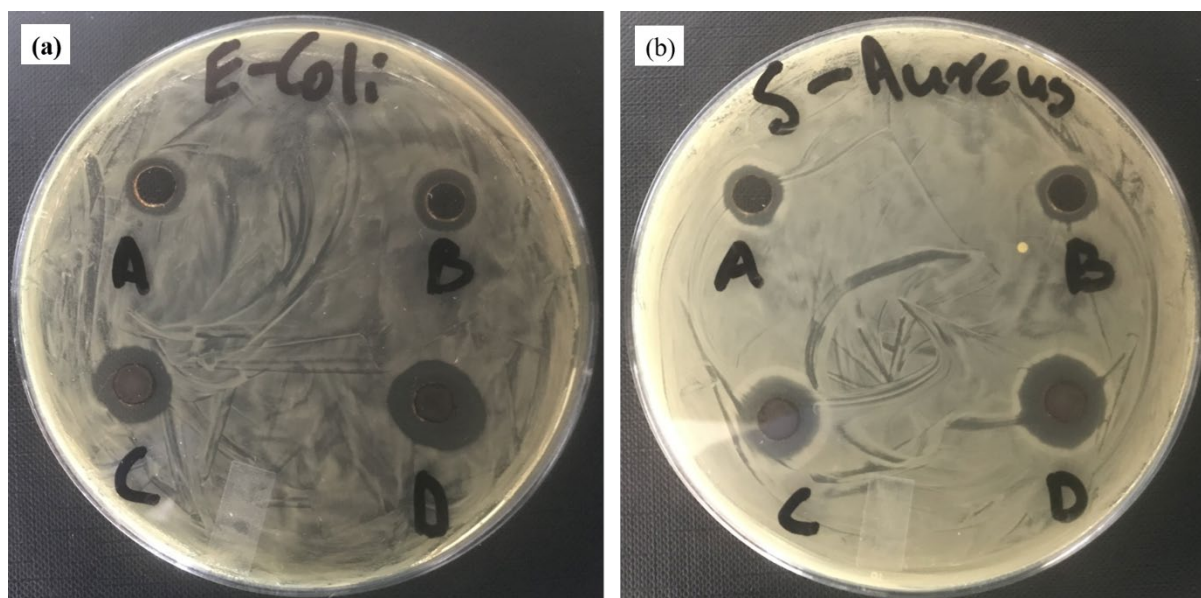


Figure 3.6: Visible inhibitory Zones produced by (A) 74 nm, (B) 48 nm, (C) 24 nm and (D) 16 nm AgNPs-DA against (a) *E. coli* and (b) *S. aureus*.

The minimum inhibitory concentrations (MIC) of AgNPs-DA of different sizes were determined using the serial dilution method. In the 8 test tubes filled with LB broth, AgNPs-DA and either *E. coli* or *S. aureus* bacterial strain, four of them appeared cloudy or turbid, while the remaining four appeared clear. The cloudy appearance in the test tubes indicates bacterial growth whereas the clear appearance shows the inhibition of bacterial growth by AgNPs-DA. Therefore, the MIC values of AgNPs-DA were obtained from the fourth clear tube. The MIC values are summarized in **Table 3.1**. AgNPs-DA with smaller sizes were found to exhibit the lowest MIC values. These results support the observations found from

the agar well diffusion method. This indicates that small-sized nanoparticles inhibit bacterial growth at lower concentrations compared to bigger sized-nanoparticles. The optical density (OD) of bacterial strains treated with AgNPs-DA of different concentrations was monitored as shown in **Figure 3.7**. To make a good comparison, the OD of untreated bacterial strains was obtained. In this case, the OD represents the concentration of bacteria. The OD of untreated bacterial strains was as high as 2 a.u which shows the high concentration of bacteria. The OD of bacterial strains treated with AgNPs-DA decreases gradually to almost zero. The OD remained almost zero as the concentration of AgNPs-DA increased. The slight increase in OD at higher concentrations can be attributed to the absorbance of AgNPs-DA. These observations were made in all AgNPs-DA sizes. It can be observed from **Figure 3.6** that the MICs of AgNPs-DA with small sizes is lower compared to those of larger sized nanoparticles. These results highlight the high efficacy of smaller sized nanoparticles compared to those of bigger sizes.

The MIC and CC₅₀ values obtained for the AgNPs-DA were further used to determine their selectivity index (SI) values. The SI value is defined as a ratio of the dose of material that causes the desired therapeutic effect to the amount that elicits toxicity (i.e. CC₅₀/MIC) and it is used to determine the toxicity/efficacy relationship of therapeutic agents. This ratio is considered as a safety margin, the wider the margin, the safer the therapeutic agent. A therapeutic agent is considered favourable if the SI value is ≥ 10 . As shown in **Table 3.1**, the SI values obtained for 16, 24, 48 and 74 nm AgNPs-DA were found to be 6, 4.1, 1.3 and 1.4, respectively, against both *E. coli* and *S. aureus*. Although these SI values are considered unfavourable, the 16 nm AgNPs-DA sample had a moderate safety profile out of the four samples tested.

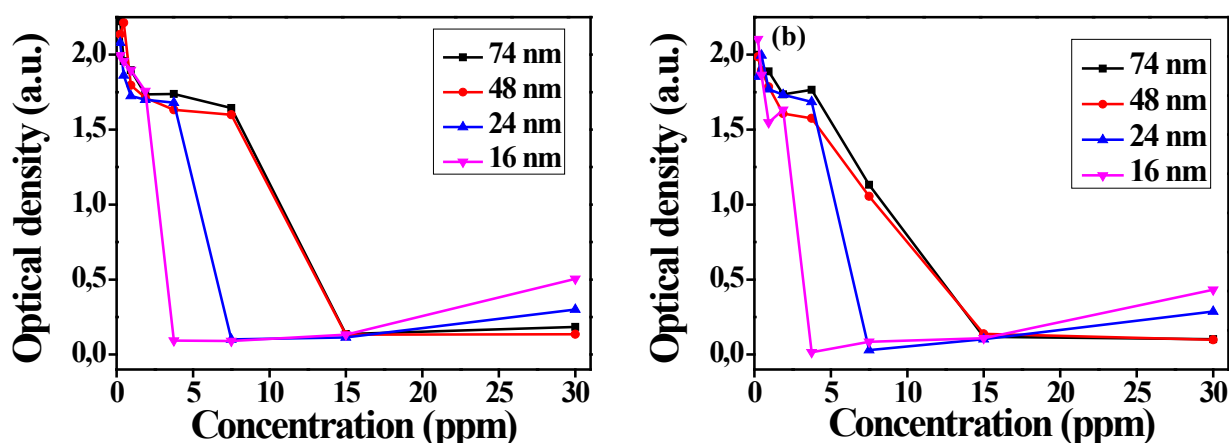


Figure 3.7: The relationship between bacterial growth (OD) and the concentration of AgNPs-DA of different sizes, tested against (a) *E. coli* and (b) *S. aureus*.

Table 3.1: Summary of the zones of inhibition, MIC, CC₅₀ and SI values of the AgNPs-DA of various sizes

Sample	Zone of Inhibition (mm)		MIC ($\mu\text{g/mL}$)		CC ₅₀ ($\mu\text{g/mL}$)	SI value	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>		<i>E. coli</i>	<i>S. aureus</i>
16 nm	17	15	3.75	3.75	22.4	6	6
24 nm	14	13	7.5	7.5	30.9	4.1	4.1
48 nm	12	10	15	15	18.9	1.3	1.3
74 nm	10	9	15	15	21.4	1.4	1.4

The bacteriostatic effects of AgNPs-DA were further evaluated. To conduct this study, bacterial solutions were treated with NPs and allowed to incubate for 14 days, with sampling every 24 h to investigate the OD of the solutions. An increase in the OD of the bacterial solution indicates an increase in the number of bacteria in the solution. **Figure 3.8** shows the bar graphs representing the bacteriostatic effects of the AgNPs-DA. The bar graphs A, B, C and D represent the bacteriostatic effects of 74 nm, 48 nm, 24 nm and 16 nm AgNPs-DA against (a) *E. coli* and (b) *S. aureus*. The bacterial growth was very low on day 1, indicating the prevention of bacterial growth by AgNPs-DA of various sizes. The OD gradually increased on day 2, with further or continuous increase as days went by for 74 nm and 48 nm AgNPs-DA. This indicates that AgNPs-DA with larger sizes lost their antibacterial activity within the first 24 h. The increase in OD is another way of showing the bacterial growth in

the samples. AgNPs-DA with smaller sizes (16 nm and 24 nm) showed an excellent bacteriostatic ability for both *E. coli* and *S. aureus*. Even after 14 days, there was no indication of bacterial growth. This simply signifies that smaller nanoparticles were able to strongly suppress bacterial growth as compared to bigger nanoparticles. It is important to note that the ODs were smaller on the *E. coli* than the *S. aureus* for the 16 nm and 24 nm AgNPs-DA, further illustrating higher sensitivity of *E. coli* as explained earlier. The ability of these AgNPs-DA materials to resist bacterial growth opens more gates for various applications such as wound healing or water purification.

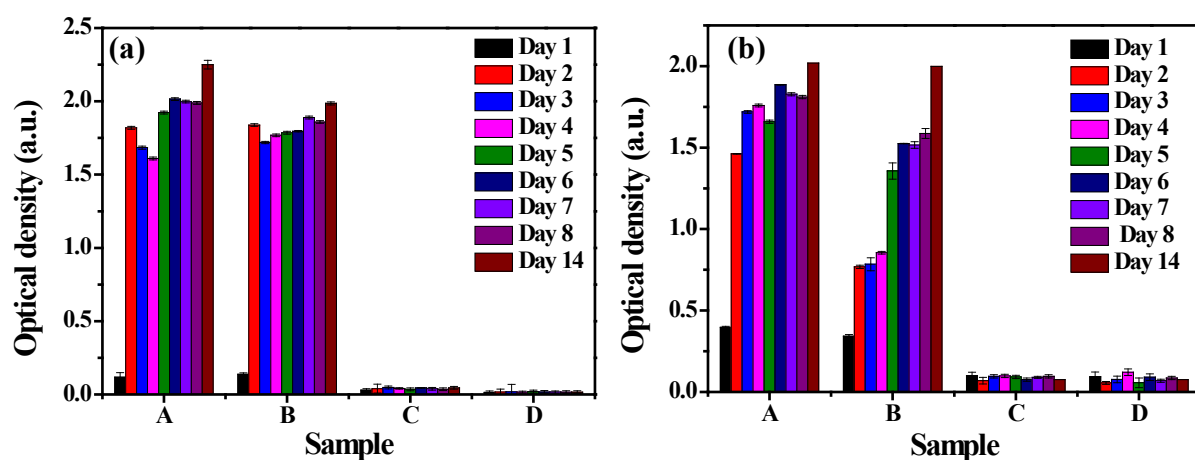


Figure 3.8: The bar graph representation of the bacteriostatic effect of (A) 74 nm, (B) 48 nm, (C) 24 nm and (D) 16 nm AgNPs-DA against (a) *E. coli* and (b) *S. aureus*.

3.4 Conclusions

This study successfully highlighted the straightforward size-controlled synthesis of AgNPs using DA as both the reducing and capping agent. The obtained AgNPs-DA were found to be spherical-like in shape as evident from TEM analysis. The optical studies confirmed the successful synthesis of AgNPs-DA as denoted by the presence of the SPR bands in the UV-vis spectra. The PXRD analysis showed that AgNPs with FCC structural phase were obtained. The cytotoxicity of the as-synthesized AgNPs-DA against BHK-21 cells was investigated. AgNPs-DA of different sizes showed concentration-dependent cytotoxicity. The cell viability of the BHK-21 cells was highly compromised by AgNPs-DA of all the sizes/diameters at concentrations above 54 $\mu\text{g/mL}$. The CC_{50} values of AgNPs-DA were fairly high, which indicates the low cytotoxic nature of AgNPs-DA against the BHK-21 cells. The synthesized AgNPs-DA were found to pose an interesting antimicrobial activity against *E. coli* and *S. aureus*. AgNPs-DA with smaller sizes were found to be more effective in

inhibiting bacterial growth compared to the bigger ones as evident from their bacterial inhibition zones. In addition, smaller AgNPs-DA were found to have lower MIC values compared to the bigger nanoparticles. Most interestingly, these AgNPs-DA can keep both *E. coli* and *S. aureus* dormant for over 14 days. In general, the antibacterial and cytotoxicity studies showed the 16 nm AgNPs-DA sample to be more potent and moderately safer compared to the bigger AgNPs-DA samples. Although efforts will still be required to improve the safety of these NPs for potential application in wound healing, the 16 nm AgNPs-DA is a promising candidate for future antibacterial wound dressings.

3.5 References

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Chapter 4

A comparison study on the antimicrobial activity of silver@polydopamine and silver/polydopamine core-shell composites

4.1 Introduction

Silver nanoparticles (AgNPs) are arguably the most significant and commonly known metal-based antimicrobial agents, even though their lethal mechanisms against pathogenic microbes are not fully understood. The antimicrobial activity of Ag has been known for centuries [1, 2]. AgNPs possess a wide spectrum of antibacterial activity due to their large surface area, their ability to easily release lethal Ag ions and the production of reactive oxygen species (ROS) [2, 3]. In addition, the ease of AgNPs to contact and penetrate the microorganisms' cell membrane gives them the advantage to attack vital organelles. Previous studies have demonstrated that AgNPs interact with sulphur-containing proteins and phosphorus-containing compounds like DNA inside the bacterial membrane [4-6]. Ideally, nanoparticles target or deactivate the respiratory chain by interfering with DNA replication and suppressing cell division, leading to cell death [7-10]. In addition, these mechanisms inhibit mutation and bacterial resistance of microbes towards AgNPs, which is one of the main concerns for most antibiotics [11].

Even though Ag has been exploited in various commercial products (e.g., food preservatives, cosmetics, and wound dressings), they still attract most research due to their practical application in various fields. However, the critical challenge of AgNPs agglomeration still exists and limits their practical applications. Various methods for the fabrication of AgNPs

have been extensively reported over the past decades [12-14]. Commonly, AgNPs are prepared by reduction of silver nitrate (AgNO_3) and stabilized by capping agents such as trisodium citrate, sodium dodecyl sulphate and cetyltrimethylammonium bromide (CTAB), and many more [15, 16, 17]. Polymers such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG) and dextran have also been used to synthesize AgNPs-based nanocomposites [18-20]. However, some of these capping agents and polymers are toxic to human cells, non-biodegradable, and therefore cannot be used for biological applications [21]. Non-toxic and biodegradable polymers have been used to fabricate and stabilize AgNPs-based nanocomposites. Polydopamine (PDA) is one of the polymers that are known to be non-toxic and biodegradable [22, 23].

PDA can easily be obtained from the self-polymerization of DA, a biomolecule of catecholamine and phenylamine families [24, 25]. Due to their inspired mussel-adhesion abilities/properties inherent from DA, PDA can easily be coated on various materials [24]. In addition, PDA can also be synthesized in the form of sheets or spheres. Furthermore, the control over the shapes, sizes and uniformity of PDA offers great advantages of using it as a template for various applications [26-29]. The high abundance of catechol and amine groups offer PDA the ability to undergo secondary reactions. These functional groups endow PDA with the potential to adsorb and reduce metal ions. Recent studies have shown the ability of PDA to reduce Ag ions to form AgNPs [30]. This facile method for fabrication and incorporation of AgNPs on the surface of PDA have been investigated [31]. It is also important to point out that PDA has proven to be biocompatible with insignificant toxicity against mammalian cells [30-33]. This gives PDA the potential to be used in the fabrication of nanocomposites that can be employed in water treatment and biological applications.

In this study, we present a facile, green and efficient approach to fabricate nanocomposites based on AgNPs and PDA. In the process, AgNPs were either coated (Ag-PDA) or incorporated on the outside (Ag@PDA) surface of PDA. For Ag-PDA, DA was firstly used to pre-reduce AgNO_3 followed by its polymerization and coating on the surface of AgNPs. A novel way of fabricating PDA-coated AgNPs is presented in this study, which involves *in situ* polymerization of DA. In addition, to fabricate Ag@PDA nanocomposites, AgNO_3 was directly reduced on the surface of PDA spheres without the use of any reductant. Due to the adherent nature of PDA, the stability of AgNPs can be improved and their toxicity reduced. The as-synthesized nanocomposites have shown a wide range of antibacterial activity against

gram-positive (*S. aureus*) and gram-negative (*E. coli*) with good biocompatibility. Their insignificant toxicity against BHK-21 cells with cell viability of over 80% at concentrations as high as 200 µg/mL was observed. Due to the bactericidal effect and non-toxic nature towards fibroblasts cells at controlled concentrations, these nanocomposites possess a great potential to be used in advanced wound dressings.

4.2 Experimental section

4.2.1 Materials

Silver nitrate (AgNO_3 <99.0%), dopamine hydrochloride, Tris buffer, Luria Bertani Broth (LB), Tryptic Soy Agar (TSA), dimethylformamide (DMF, 99.9%), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS) and penicillin-streptomycin were all purchased from Sigma Aldrich and used as received. All glassware was washed with an aquaregia solution and rinsed with distilled water several times.

4.2.2 Synthesis of nanocomposites materials

4.2.2.1 Preparation of AgNPs coated/encapsulated in PDA (Ag-PDA)

To Synthesize AgNPs coated/encapsulated in PDA, various amounts of AgNO_3 were prepared in 100 mL distilled water. The solution was heated to a boil, and 1 mL of DA hydrochloride (1 mM) was added dropwise. The colour of the solution changed immediately, from clear to pale yellow indicating that the formation process of colloidal AgNPs was initiated. The reaction was stopped after 7 minutes and cooled at room temperature. Thereafter, 50 mL of the colloidal AgNPs was withdrawn and added to 100 mL Tris-buffer (0.5 M). The mixture was stirred for 30 minutes before the addition of 10 mL dopamine hydrochloride (0.1 g). The reaction was carried out for 24 hours with gentle stirring. The product was washed 3 times with distilled water with the aid of centrifugation at 15000 rpm for 15 min.

4.2.2.2 Preparation of AgNPs on the surface of PDA (Ag@PDA)

PDA spheres were prepared by mixing 50 mL distilled water and 25 mL ethanol. To the mixture, 2 mL ammonium hydroxide (30-33%) was added and the reaction was gently stirred for 30 minutes. A 0.1 g DA was dissolved in distilled water and added to the mixture. The reaction was carried out for 30 hours and the product was washed with distilled water with the aid of a centrifuge at 15000 rpm for 15 minutes before characterization. AgNPs loaded on PDA spheres were prepared by dispersing 10 mg of PDA spheres in 30 mL distilled water through ultrasonication in ice-cold water. Various amounts of AgNO_3 (1, 5 and 10 mM) were prepared and added (10 mL) to the PDA suspension. The reaction was carried out for 30

minutes. The product was washed using centrifugation at 15000 rpm for 15 minutes then dried at room temperature before characterization.

4.2.3 Cell viability studies

Please refer to section 3.2.3 for detailed procedure. The final concentrations of the nanocomposites (Ag@PDA and Ag-PDA) on the plates were ranging between 31 – 1000 µg/mL.

4.2.4 Antimicrobial activity tests

4.2.4.1 Well diffusion method

Please refer to section 3.2.4.1 for detailed procedure.

4.2.4.2 Minimum inhibitory concentration (MIC) and bacteriostatic studies

Please refer to section 3.2.4.2 for detailed procedure. The concentration range of the nanocomposites was 100 to 0.78125 µg/mL from the first to the last tube.

4.2.5 Characterization

All excess impurities from colloidal solutions were removed using a universal 320R centrifuge at a maximum speed of 15000 rpm for 15 minutes. Chemical structure analysis was done using a Bruker Tensor 27 Fourier transform infrared (FTIR) spectrometer. Thermal analysis was conducted using a Perkin Elmer TGA 6000 thermogravimetric analyser (TGA) using high-purity nitrogen at a heating rate of 10 °C/min and gas flow rate of 10 mL/min. Transmission electron microscopy (TEM) images were obtained from FEI Tecnai-T12 microscopy at an electron acceleration voltage of 120 kV and beam spot size of 10-100 nm. The TEM particle diameters were determined using ImageJ software. Powdered X-ray diffraction (PXRD) analysis was performed on a Bruker D2 Phaser with Cu radiation and each scan was recorded over 20 min.

4.3 Results and discussion

4.3.1 Characterization of nanocomposites

AgNPs and PDA based nanocomposites were successfully synthesized through facile processes. These processes involved self-polymerization of DA at room temperature with gentle stirring to produce PDA spheres through polymerization of DA. To polymerize DA, mild alkaline conditions were required. Therefore, ammonium hydroxide and tris buffer solutions were both used to initiate the polymerization process. To produce the nanocomposites, AgNPs were either encapsulated inside (Ag-PDA) or incorporated outside the surface (Ag@PDA) of PDA, *in-situ*. The morphologies and sizes of the as-synthesized

PDA and the nanocomposites were studied using TEM, and their micrographs are respectively shown in Figure S1 and **Figure 4.1**.

Monodispersed PDA spheres with an average diameter of 238 nm were obtained as shown in **Figure S4.1** (A and B). **Figure 4.1** (a-c) and (d-f) respectively shows the TEM micrographs of Ag-PDA and Ag@PDA nanocomposites prepared using various concentrations of AgNO₃. The size distributions of AgNPs encapsulated inside (Ag-PDA) and incorporated outside (Ag@PDA) PDA are presented in **Figure S4.2** (a-c) and (d-f). The average diameters of spherical-like AgNPs were found to be 4.1, 8.4 and 8.8 nm for Ag-PDA nanocomposites prepared with 1, 5 and 10 mM AgNO₃, as indicated in **Figure 4.1a, b and c**, respectively. The encapsulation of AgNPs inside PDA resulted in the formation of Ag-PDA core-shells as witnessed in **Figure 4.1(a-c)**. The thickness of the PDA surrounding AgNPs was not uniform, with the thickness sizes ranging from 2 to 7 nm. **Figures 4.1d, e and f** respectively show the TEM micrographs of Ag@PDA nanocomposites prepared with AgNO₃ concentrations of 1; 5 and 10 mM. AgNPs incorporated on the surface of PDA to form Ag-PDA were also found to be spherical. In addition, uniformly dispersed AgNPs on the surface of PDA spheres with the average diameters 8.1, 9.1 and 12.7 were obtained. These diameters correspond to 1-, 5- and 10-mM concentrations of AgNO₃ used in the preparation of Ag-PDA as shown in **Figures 4.1d, e and f**, respectively. The average diameter slightly increased with an increase in the concentration of AgNO₃.

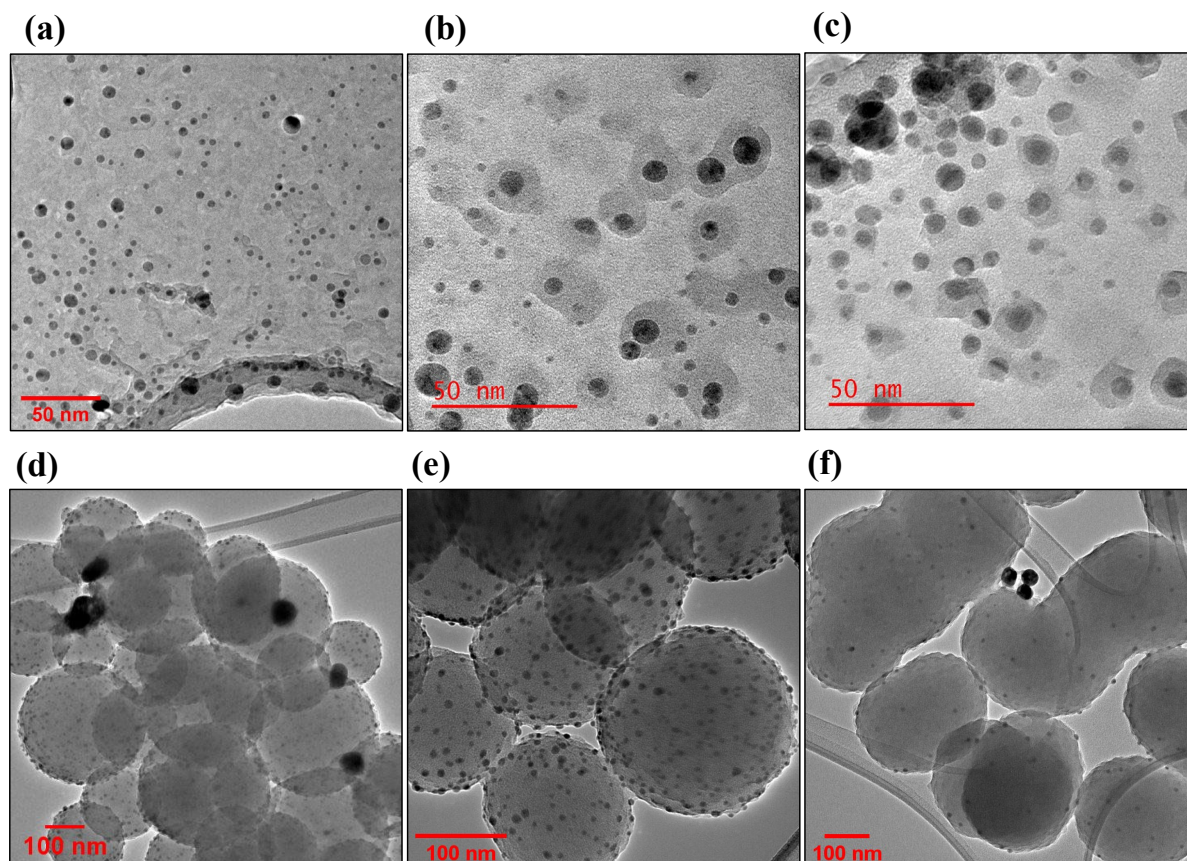


Figure 4.1: TEM images of (a-c) Ag-PDA and (d-f) Ag@PDA nanocomposites at different AgNO_3 concentrations. [(a, d) 1 mM, (b, e) 5 mM and (c, f) 10 mM].

The crystal phase and crystallinity of PDA, Ag-PDA and Ag@PDA were investigated using PXRD as shown in **Figure 4.2**. The diffractogram of pristine PDA spheres used to fabricate Ag@PDA nanocomposite shown in **Figure S4.3** reveals a broad peak between 10° and 30° 2θ angles. This finding is attributed to the amorphous nature of PDA. It is important to note that the synthesis of Ag-PDA nanocomposite was performed *in-situ*, therefore there is no diffraction pattern for pristine PDA. **Figure 4.2** (a & b) respectively show the diffraction patterns of Ag-PDA and Ag@PDA nanocomposite prepared with different amounts of Ag loading. Diffractograms i, ii and iii represent the nanocomposites prepared with 1, 5 and 10 mM of AgNO_3 , respectively. The crystalline diffraction peaks at 2θ angles of 38.6° , 44.7° , 64.7° , 77.7° and 81.9° for Ag-PDA and 39.1° , 45.2° , 65.1° , 78.3° and 82.4° for Ag@PDA were observed. These peaks correspond to the diffraction of (111), (200), (220), (311) and (222) lattice planes of face centred cubic (FCC) phase of Ag crystal (PDF: 01-071-4612). The presence of characteristic diffraction peaks of Ag crystal in both samples provides evidence that PDA and its monomer, DA, were able to reduce Ag ions to metallic silver. The PDA

amorphous peak was still observable between 10° and 30° 2θ angles in some of the diffractograms. It was also observed that the diffraction peaks did not shift, and the widths of the peaks were almost the same after varying the concentration of AgNO_3 . This can be attributed to the fact that there was no huge difference in the sizes of AgNPs as was observed from TEM analysis. The presence of unassigned peaks (marked with an asterisk) on the diffractograms of Ag-PDA prepared with 10 mM, and that of Ag@PDA prepared with 5 mM, and 10 mM of AgNO_3 were observed. These peaks were matched to AgCl crystal (* PDF: 00-031-1238). The formation of AgCl crystal can be attributed to the interaction of excess Ag ions from AgNO_3 and Cl ions from dopamine hydrochloride.

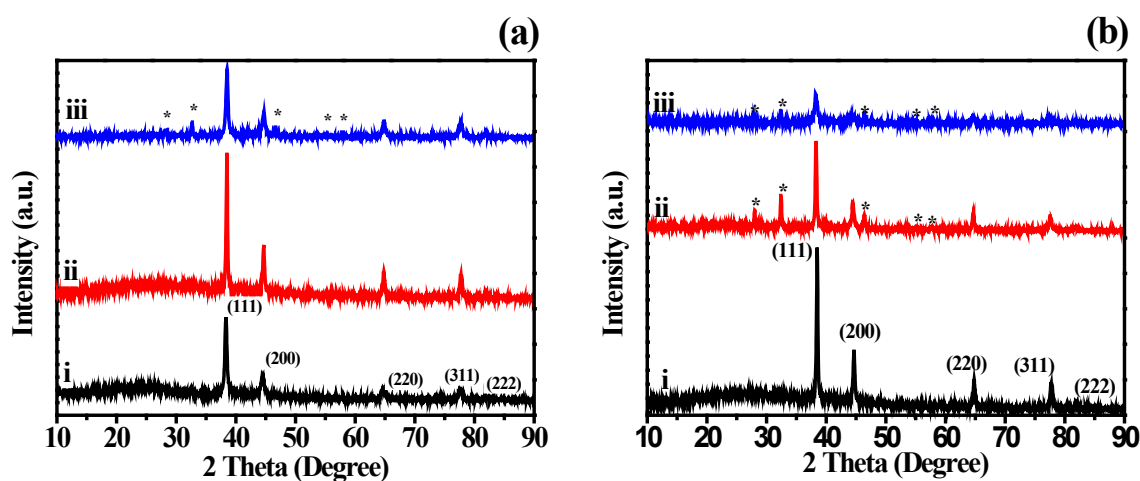


Figure 4.2: PXRD patterns of (A) Ag-PDA and (B) Ag@PDA nanocomposites prepared with different concentrations of AgNO_3 [(i) 1 mM, (ii) 5 mM and (iii) 10 mM].

Chemical structures of DA (DA), PDA, Ag-PDA and Ag@PDA nanocomposites were investigated using FT-IR spectroscopy as shown in **Figure 4.3**. The FT-IR spectra show well-defined characteristic functional groups of DA as indicated in **Figure 4.3a(i)** and **3b(i)** respectively. The broad vibrational band around 3250 cm^{-1} shows the overlapping stretching modes of $-\text{OH}$ and $-\text{NH}$ groups. The sharp peaks around 1700 cm^{-1} and 1250 cm^{-1} are attributed to $\text{C}=\text{O}$ and $\text{C}-\text{O}$ vibrations respectively, while the peak at 1500 cm^{-1} represents the vibrational modes of $\text{C}=\text{N}$ and $\text{C}=\text{C}$. The PDA, Ag-PDA and Ag@PDA spectra are shown in **Figure 4.3a(ii-v)** and **3b(ii-v)** displayed almost all vibrational bands that are present in the pristine DA spectra. The two sets of spectra have aided in elucidating the interaction between Ag and PDA. The bands of Ag-PDA and Ag@PDA nanocomposites are broadened and slightly shifted to the higher wavenumber compared to that of PDA. The broadening and the slight shifting of the bands could be the indication of the interaction between Ag and PDA

through hydrogen bonding. This interaction promotes the attachment and stability of AgNPs on the surface of PDA. There was no noticeable change in the spectra when the concentration of AgNO₃ was changed. This shows that the incorporation of AgNPs does not affect the chemical structure of PDA.

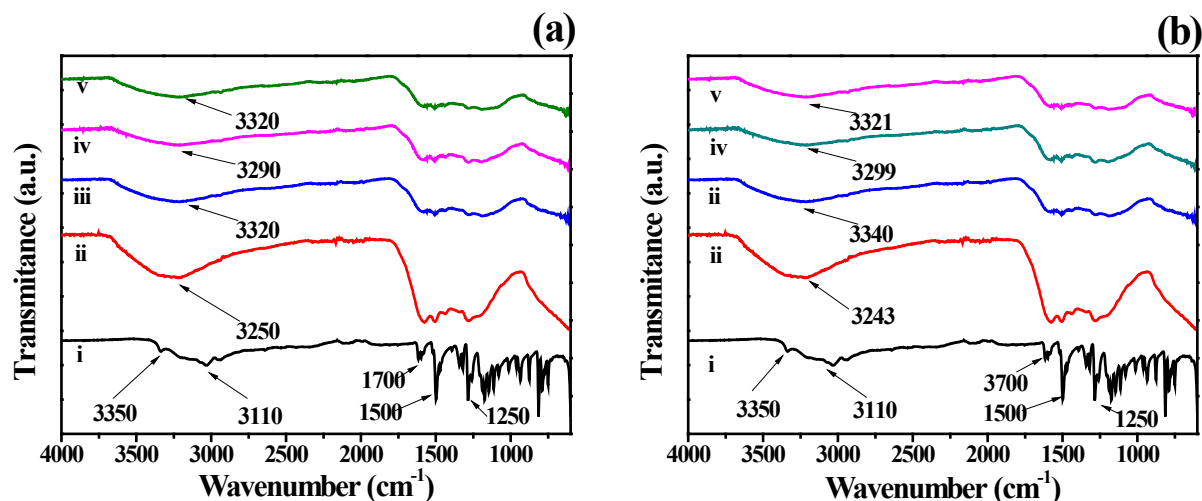


Figure 4.3: FTIR spectra of (a) Ag-PDA and (b) Ag@PDA nanocomposites at different AgNO₃ concentrations [(i) DA, (ii) PDA, (iii) 1 mM, (iv) 5 mM and (v) 10 mM].

The thermal stability study of the materials was carried out using thermogravimetric analysis (TGA) within the temperature range of 35 – 900 °C. The TGA thermograms of Ag-PDA and Ag@PDA are respectively shown in **Figure 4.4(a & b)** and their derivative-TGA (D-TGA) thermograms in **Figure S4.4(a, b & c)**. The multi-step decomposition of PDA can be easily observed from the D-TGA curves. This is a result of the presence of different chemical groups and the heterogeneous structure of PDA [26]. The first decomposition occurs around 50°C and can be attributed to the removal of residual moisture. The other decomposition peaks occurred around 360 °C, 510 °C and 620 °C, representing the significant loss of the polymer backbone and functional groups. The decomposition around 360 °C was due to the loss of the side groups of the polymer such as hydroxyl (-OH) and amine (-NH). Between 510 °C and 620 °C, the polymer completely decomposes due to the cleavage of the C-C bonds of the polymer backbone. It was, however, observed that the decomposition of PDA was accelerated due to the incorporation of AgNPs. This can be seen by the shift in the decomposition peaks towards lower temperatures in Figure 4(a and b). AgNPs could be acting as a catalyst, causing the rapid decomposition of PDA. The total residues of 10, 15 and 20% for Ag-PDA and 5, 8 and 17% for Ag@PDA prepared with varying AgNO₃ concentrations were observed. The residue confirms the presence and the amount of Ag

loaded on the PDA. The amount of residue increased with an increase in the concentration of AgNO_3 used.

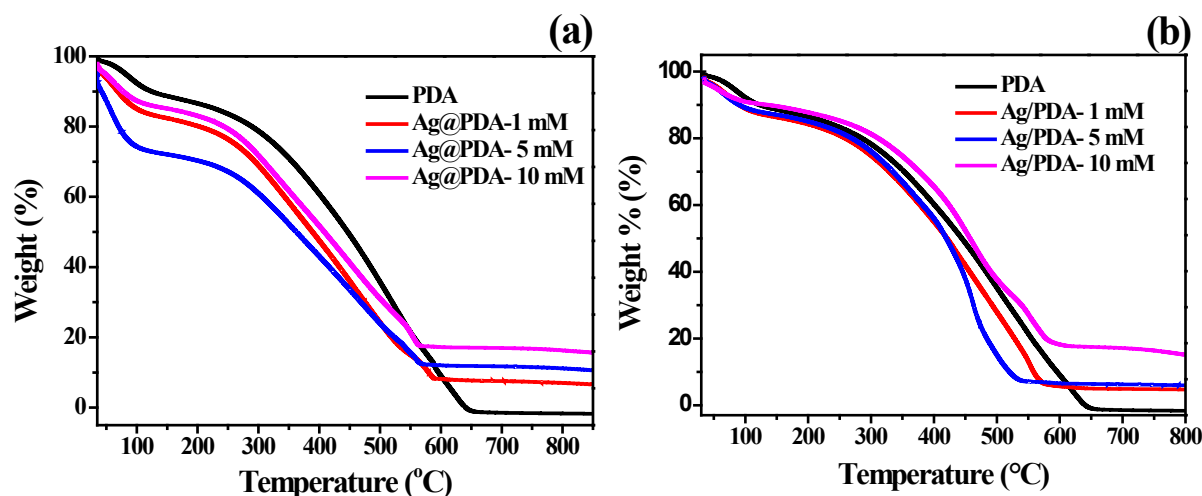


Figure 4.4: TGA of (a) Ag-PDA and (b) Ag@PDA nanocomposites at different AgNO_3 concentrations.

4.3.2 Biocompatibility studies of nanocomposites

The biocompatibility of Ag-PDA and Ag@PDA at different concentrations was tested by evaluating their cytotoxicity towards BHK-21 cells *in vitro*. The cell viability was determined using the MTS cell proliferation assay after being treated with each sample. Furthermore, the effect of Ag loading (in terms of the AgNO_3 concentration) on the cytotoxicity was also evaluated. As shown in **Figure 4.5**, both Ag-PDA (a) and Ag@PDA (b) did not show a significant level of cytotoxicity towards both BHK-21 cells, especially at low concentrations. Ag-PDA did not show significant toxicity towards the cells, even at concentrations as high as $250 \mu\text{g/mL}$, with cell viability exceeding 50% for all the Ag loadings (i.e. 1, 5 and 10 mM AgNO_3). Ag@PDA displayed significant signs of toxicity to the cells at higher concentrations compared to Ag-PDA. This is attributed to the fact that AgNPs are encapsulated inside PDA for Ag-PDA whereas AgNPs are supported on the surface of PDA in Ag@PDA, making them directly exposed to the cells. Since AgNPs are encapsulated inside PDA, the release of Ag ions and the direct contact to the cells is limited, hence low toxicity due to Ag-PDA. The amount of Ag loading on the PDA also showed to influence the cytotoxicity. These observations were clear on the Ag-PDA than on the Ag@PDA samples which did not show a clear trend. At high concentrations of Ag loading (10 mM), Ag-PDA proved to be more toxic to the cells. The cell viability of the BHK-21 cells was above 80% at lower loading of Ag (i.e., 1 and 5 mM AgNO_3) between the concentration range of 31 to 250

$\mu\text{g/mL}$. As the loading concentration increased to 10 mM AgNO_3 , a notable decrease in cell viability was observed, with the lowest concentration of 31 $\mu\text{g/mL}$ showing cell viability of $\sim 60\%$.

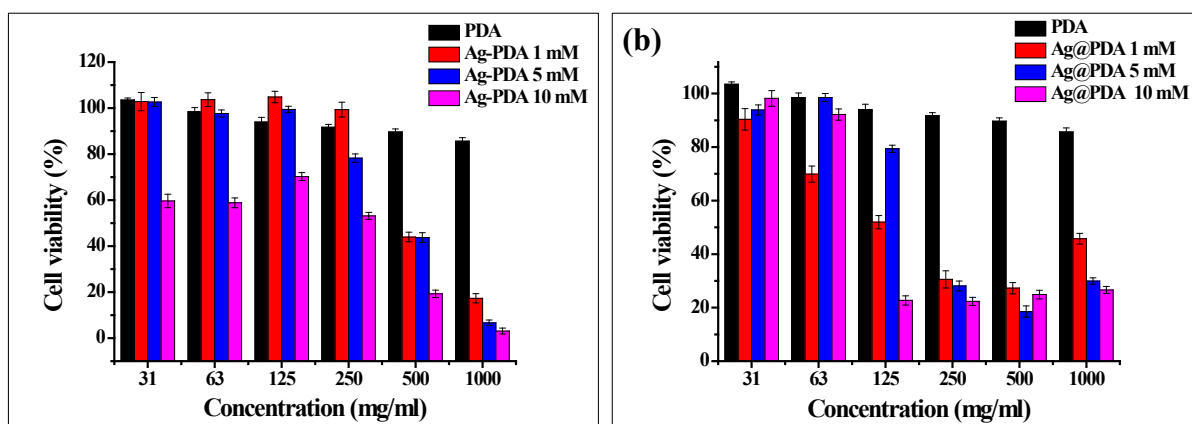


Figure 4.5: Cell viability of BHK-21 cells exposed to (a) Ag-PDA and (b) Ag@PDA at different concentrations of AgNO_3 .

The cytotoxicity effects of Ag-PDA and Ag@PDA towards the BHK-21 cells was further evaluated by determining the minimum concentration at which they kill 50% of the cells, also known as 50% cytotoxic concentration (CC_{50}). The CC_{50} values of Ag-PDA and Ag@PDA against BHK-21 cells were determined from the sigmoid curves in **Figure S4.5** (a & b) and are recorded in **Table 4.1**. The obtained CC_{50} values were relatively high indicating that both Ag-PDA and Ag@PDA are less toxic towards the cells. Ag-PDA had higher CC_{50} values compared to Ag@PDA, which is in support of the cell viability findings. The CC_{50} values of Ag-PDA were observed to be high for low loading of Ag, a phenomenon that was also observed in the cell viability assay.

Table 4.1: Summary of the CC_{50} values obtained for Ag-PDA and Ag@PDA nanocomposites prepared with different concentrations of AgNO_3 (A) 1 mM, (B) 5 mM (C) 10 mM, and (D) PDA

Sample	CC_{50} ($\mu\text{g/mL}$)	
	Ag-PDA	Ag@PDA
A	476	231
B	430	210
C	400	180
D	-	-

4.3.3 Antimicrobial activity of nanocomposites

The antimicrobial activity of the nanocomposites was determined by a standard well-diffusion method against gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria. The qualitative antimicrobial activity was done by monitoring the ability of each material to inhibit bacterial growth. The zones of bacterial inhibition were photographed in the agar plates and are shown in **Figure 4.6** (a and b) for Ag-PDA and Ag@PDA nanocomposites, respectively. The nanocomposites proved to possess antimicrobial properties as indicated by clear zones of inhibition around the wells on the agar plates. A slight increase in the diameter of the zones of inhibition in response to an increase in the concentration of AgNPs loaded on PDA was observed for both bacterial strains. These observations were clearer in Ag@PDA compared to Ag-PDA samples. Additionally, the activity of Ag@PDA was deduced to be higher compared to Ag-PDA against both *E. coli* and *S. aureus*. Since AgNPs are supported on the outside surface of PDA for Ag@PDA, it becomes easy for them to penetrate, interact and destroy the vital cell organelles of neighbouring bacterial cells. AgNPs in Ag-PDA on the other hand, were not in direct contact with the bacterial cell wall since they are encapsulated inside the PDA, which reduces their antimicrobial activity. It is important to note that the pristine PDA did not show any antimicrobial activity against both bacterial strains as there was no clear zone of inhibition observed. The measured zones of inhibition for Ag-PDA and Ag@PDA nanocomposites are recorded in **Tables 4.2 and 4.3**.

To further investigate the antimicrobial activity of the nanocomposites, the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) of Ag-PDA and Ag@PDA were determined. The MIC and MBC values of Ag-PDA and Ag@PDA are respectively summarized in **Tables 4.2 and 4.3**. Equal MIC values of 50 µg/mL for all Ag-PDA samples with different Ag loading (**Table 4.2** A, B and C) were observed in both *E. coli* and *S. aureus*. The MIC values obtained for Ag@PDA samples were lower compared to those of Ag-PDA shown in **Table 4.3**. The MIC values were found to decrease with an increase in the concentration of AgNPs loading as indicated in **Table 4.3** (A, B and C) for 1, 5 and 10 mM AgNO₃ used. These findings signify the requirement of less amount of Ag@PDA to inhibit bacterial growth compared to Ag-PDA. MIC values of as low as 3.125 µg/mL were obtained from the Ag@PDA sample prepared with 10 mM AgNO₃. The ability of the nanocomposites to kill the bacterial cells was investigated by determining the MBC values of the samples. The MBC was obtained by swabbing each of the bacterial strains

treated with the nanocomposites on the agar plates shown in **Figure S4.6** (a and b) for Ag-PDA and Ag@PDA, respectively. Bacterial growth was observed within 24 hours of swabbing the Ag-PDA nanocomposites plate. This is an indication that Ag-PDA nanocomposites could only inhibit bacterial growth and were unable to kill the bacterial cells. Ag@PDA nanocomposites prepared by 10 mM AgNO₃ [**Figure S4.6b(C)**] exhibited bactericidal properties with the MBC value of 3.125 µg/mL [**Table 4.3(C)**] for both *E. coli* and *S. aureus*. When the AgNO₃ concentration was decreased to 5 mM [**Figure S4.6b(B)**], Ag@PDA only exhibited the bactericidal activity against *E. coli* with the MBC of 6.25 µg/mL [**Table 4.3(B)**]. *S. aureus* was found to be resistant at this concentration of AgNO₃ since no bacterial cell death was observed after 24 hours of swabbing. Ag@PDA prepared with 1 mM AgNO₃ also did not show any evidence of causing bacterial cell death [**Figure S4.6b(A)**] for both *E. coli* and *S. aureus*.

The MIC and CC₅₀ values obtained for the Ag-PDA and Ag@PDA nanocomposites were further used to determine the selectivity index (SI) values of these materials. The SI value is defined as a ratio of the dose of material that causes the desired therapeutic effect to the amount that elicits toxicity (i.e., CC₅₀/MIC) and it is used to determine the toxicity/efficacy relationship of therapeutic agents. This ratio is considered as a safety margin, the wider the margin, the safer the therapeutic agent. A therapeutic agent is considered favourable if the SI value is ≥ 10 . As shown in **Table 4.2**, the SI values obtained for the Ag-PDA nanocomposites prepared with 1, 5 and 10 mM AgNO₃ concentrations were found to be 9.5, 8.6 and 8.0, respectively, against both *E. coli* and *S. aureus*. **Table 4.3** on the other hand shows that the Ag@PDA nanocomposites prepared with 1, 5 and 10 mM AgNO₃ concentrations had SI values of 37.0, 33.6 and 57.6, respectively, against *E. coli*, and 18.5, 16.8 and 57.6, respectively, against *S. aureus*. These results show that the Ag-PDA samples had moderate safety margins (close to 10), while the Ag@PDA nanocomposites showed favourable safety profiles at all the tested AgNO₃ concentrations.

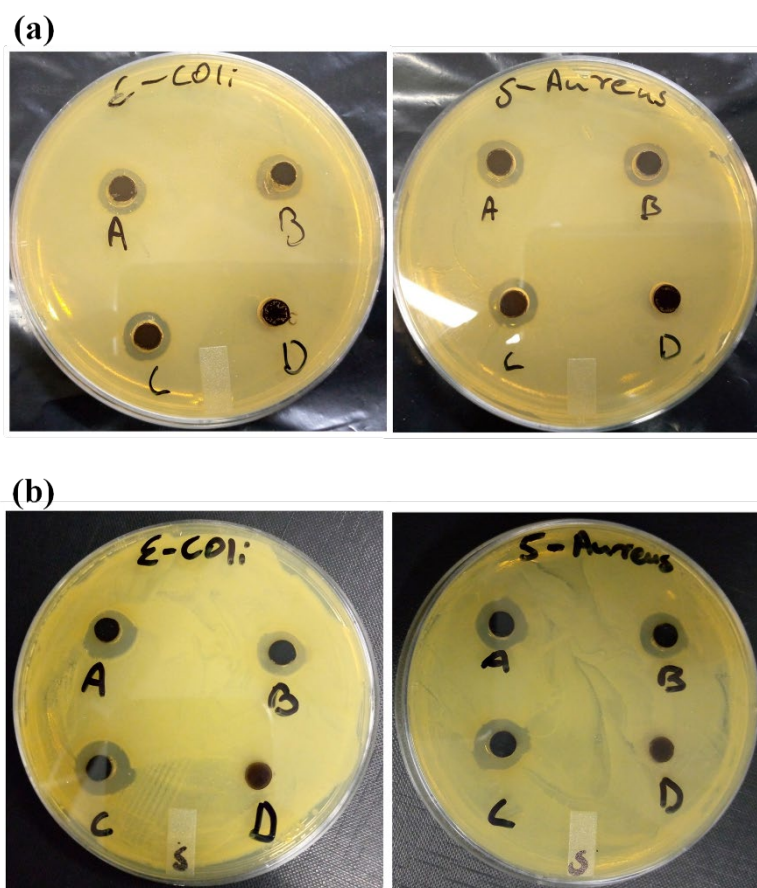


Figure 4.6: Inhibitory Zones photographs produced by (a) Ag-PDA and (b) Ag@PDA nanocomposites at different AgNO_3 concentrations [(A) 1 mM, (B) 5 mM and (C) 10 mM] and (D) PDA

Table 4.2: Summary of the zone of inhibition, MIC, MBC and SI values of Ag-PDA nanocomposite prepared with different concentrations of AgNO_3 (A) 1 mM, (B) 5 mM (C) 10 mM, and (D) PDA

Sample	Zone of Inhibition (mm)		MIC ($\mu\text{g/mL}$)		MBC ($\mu\text{g/mL}$)		SI value	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
A	11	11	50	50	-	-	9.52	9.52
B	12	11	50	50	-	-	8.6	8.6
C	12	12	50	50	-	-	8.0	8.0
D	0	0	-	-	-	-	-	-

Table 4.3: Summary of the zone of inhibition, MIC, MBC and SI values of Ag@PDA nanocomposite prepared with different concentrations of AgNO₃(A) 1 mM, (B) 5 mM (C) 10 mM, and (D) PDA

Sample	Zone of Inhibition (mm)		MIC (µg/mL)		MBC (µg/mL)		SI value	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
A	12	11	6.25	12.5	-	-	37.0	18.5
B	13	11	6.25	12.5	6.25	-	33.6	16.8
C	15	14	3.125	3.125	3.125	3.125	57.6	57.6
D	0	0	-	-	-	-	-	-

The antimicrobial activity of the nanocomposites was also evaluated by studying the bacterial growth kinetics in the medium (LB Broth) containing each bacterial strain (*E. coli* and *S. aureus*) and the nanocomposites. The media containing only the bacterial cells and PDA were respectively used as controls. The bacterial growth was examined by measuring the optical density (OD) of the cell suspension at 600 nm. The growth kinetics were studied at a concentration range of 3.125 - 100 µg/mL for both Ag-PDA and Ag@PDA prepared with different concentrations of AgNO₃ as shown in **Figure 4.7**. **Figure 4.7** (a and b) represents the bacterial growth curves obtained for Ag-PDA prepared with 10 mM of AgNO₃ against *E. coli* and *S. aureus*, respectively. An instantaneous bacterial growth was observed within the first hour of incubation for the control samples. There was no significant difference between the suspension containing PDA and those containing the bacterial cells only (*E. coli* and *S. aureus*). This was an indication that PDA alone could not inhibit the bacteria from growing. At low concentrations of the nanocomposites (between 3.125 and 25 µg/mL), Ag-PDA inhibited *E. coli* from growing in the first 2 hours as shown in **Figure 4.7a**. The bacterial (*E. coli*) growth was indicated by the increase of OD readings and the turbidity of the suspension. The bacteria continued to grow with time as witnessed by the increasing turbidity and OD readings. When the concentration of Ag-PDA was increased to 50 and 100 µg/mL, inhibition of bacterial growth was observed throughout the testing period of the study. Ag-PDA, however, showed weaker antimicrobial activity against *S. aureus*, compared to *E. coli*, as

indicated in **Figure 4.7b**. *S. aureus* started to grow within the first hour of incubation as was evident from increasing OD readings and the turbidity of the suspensions. At lower concentrations of AgNPs loading (i.e., 1 and 5 mM AgNO₃), a similar trend of bacterial growth was observed for Ag-PDA against both bacterial growths as shown in **Figure S4.7** (a-d).

On the other hand, Ag@PDA nanocomposites were found to be able to inhibit both bacterial strains (*E. coli* and *S. aureus*) from growing as shown in **Figures 4.7c** and **d**. The OD readings were constant throughout and there was no turbidity observed in the suspensions. These results further prove the strong antimicrobial activity of Ag@PDA over that of Ag-PDA. At low concentrations of AgNPs loading (i.e., 1 and 5 mM AgNO₃) as shown in **Figure S4.7** (e-h), bacterial growth (*E. coli*) was observed within the first and the fourth hour for 3.125 µg/mL Ag@PDA. At concentrations higher than 3.125 µg/mL, the growth of the bacterial cells was retarded the entire time. However, *S. aureus* seemed to be more resistant even at concentrations as high as 25 µg/mL since bacterial growth was observed within an hour of incubation. Only when the concentration of Ag@PDA was increased to 50 and 100 µg/mL did the bacterial growth get retarded for the entire study time. It was noticed that *E. coli* is more sensitive to both Ag-PDA and Ag@PDA nanocomposite than *S. aureus*. This may be attributed to the fact that gram-positive bacteria (*S. aureus*) have a thick cell membrane that is stable compared to that of gram-negative bacteria (*E. coli*). Gram-negative bacteria are thus more susceptible to AgNPs as compared to gram-positive.

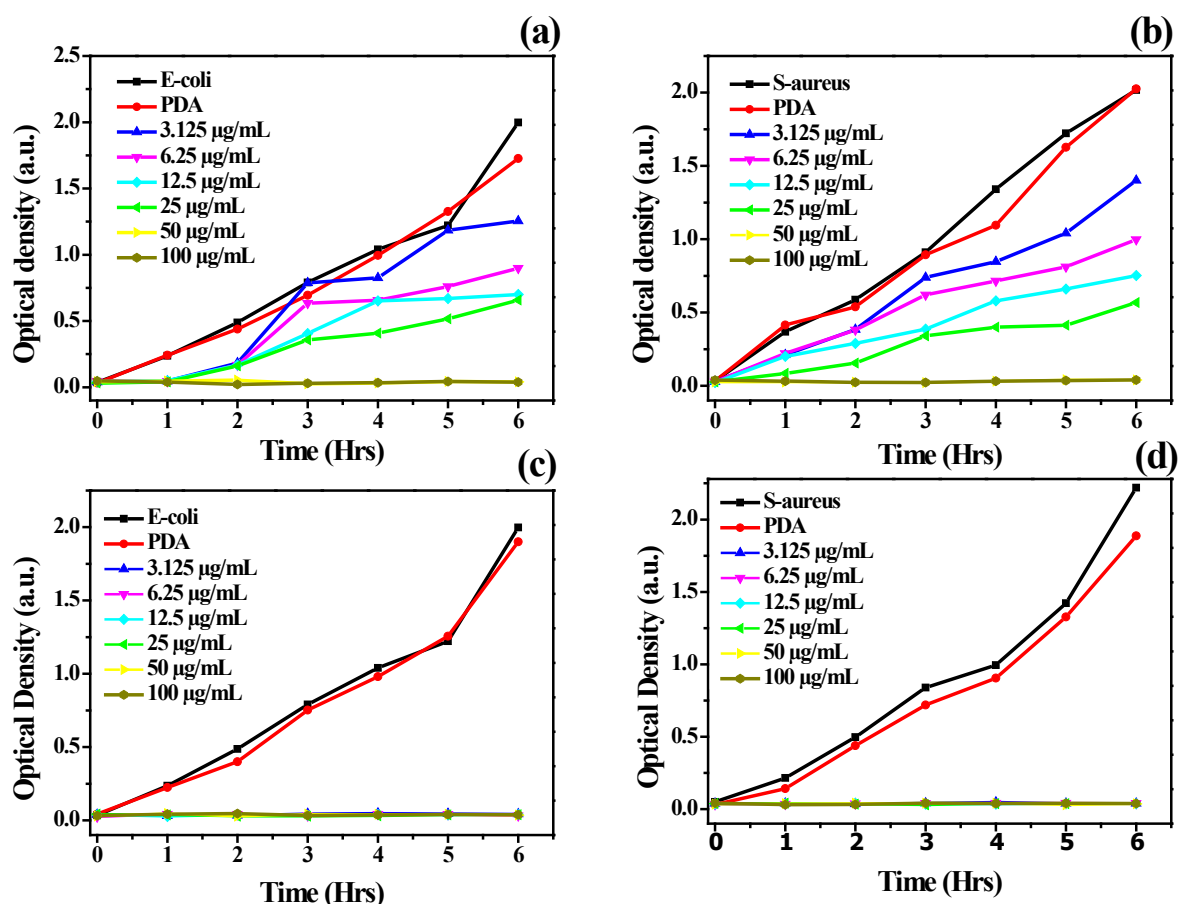


Figure 4.7: The bacterial growth curves of Ag-PDA (a, b) and Ag@PDA (c, d) against *E. coli* and *S. aureus*.

4.4 Conclusions

Ag-PDA and Ag@PDA nanocomposites were successfully produced through a facile and green synthesis method. The diameters of AgNPs were controlled by varying the concentration of AgNO₃. As the concentration of AgNO₃ increased, the diameter of the AgNPs also increased. The agglomeration and stability of AgNPs were achieved by either coating or incorporating them on the surface of PDA. Both the nanocomposites have shown excellent thermal stability as evident from the TGA. However, the thermal stability of PDA was slightly compromised because of AgNPs incorporation. Furthermore, both Ag-PDA and Ag@PDA nanocomposites showed moderate toxicity towards BHK-21 fibroblast cells. Also evident from the results obtained in this study is that the Ag-PDA and Ag@PDA nanocomposites showed inhibition of bacterial growth at concentrations that are notably lower than their minimum cytotoxic concentrations. The MIC values obtained for the Ag-PDA nanocomposites were between 8- and 10-fold lower than the CC₅₀ values, while MIC

values obtained for the Ag@PDA were between ~6- and 26-fold lower than the CC₅₀ values. These results show that these materials could potentially be used as antimicrobial agents without eliciting toxicity against mammalian cells. This phenomenon was further proved by the SI values obtained for these materials, where the Ag-PDA nanocomposites had moderate safety margins, while Ag@PDA nanocomposites had very good safety margins. The antimicrobial activity of these composites against *E. coli* and *S. aureus* was excellent. It was found that the amount of AgNPs loading in terms AgNO₃ had more effect on the antimicrobial activity of the nanocomposites compared to their sizes. At high Ag loadings, the antimicrobial activity was also high. Ag@PDA nanocomposites were more active than Ag-PDA. Moreover, Ag@PDA nanocomposites were found to be bactericidal at a high concentration of Ag loading, wherein Ag-PDA nanocomposites were only bacteriostatic. With these findings, it is clear that the synthesized nanocomposites, particularly Ag@PDA, have great potential to be used in advanced wound care treatments. This is because of their ability to kill bacteria at low concentrations and their good biocompatibility towards fibroblasts cells. Fibroblasts are the main cells present in skin connective tissue (dermis) and play a crucial role during cutaneous wound healing.

4.5 References

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Chapter 5

Improved antimicrobial activity and reduced toxicity of copper through synergistic effect of dopamine capped copper/copper oxide nanoparticles and their potential in wound healing

5.1 Introduction

The use of nanomaterials in applications such as photovoltaics, catalysis, diagnostics and therapeutics continues to gain vast research attention [1-4]. This is a result of everyday developments of new synthetic procedures that can be used to modify properties of nanomaterials to fit a specific application [1-6]. Copper (Cu) and Cu-based nanomaterials, amongst others, have attracted great research attention due to their optical, electronic, high surface area, high thermal and mechanical stability, catalytic and antimicrobial properties [1-8]. In addition, Cu is widely available, which makes it relatively cheap and easily accessible. As a result, Cu and Cu oxide (CuO/ Cu₂O) nanoparticles have been applied as one of the main ingredients in various applications including inks, polymers or packaging plastics [9, 10]. Furthermore, these NPs have also been used as antimicrobial agents in textile, and various skin products including wound dressings [11-14]. Recent studies have showcased various methods for synthesizing Cu and Cu oxide NPs. Synthetic methods such as micro-emulsification, thermal decomposition, microwave and chemical reduction among others have been widely investigated [14-17]. Chemical reduction is one of the most preferred methods for NPs synthesis due to the ease of controlling their sizes, shape and yields. This method involves the reduction of Cu salt using a reducing agent. Moreover, this method requires simple reaction conditions, promotes fast reaction time and it is relatively cost effective. The effect of Cu salts (copper acetate, copper chloride, and copper sulphate) and reducing agents (sodium hydroxide and ascorbic acid) on the sizes and shapes of Cu NPs have been previously investigated [18]. Cu and CuO NPs synthesized with different Cu salts and reducing agents yields NPs with various shapes and sizes, which is important in modifying NPs properties.

The antimicrobial activity of Cu and Cu oxide NPs has also been well studied. These NPs have proven to be very interesting biomaterials due to their broad antimicrobial and antifungal spectrum [19-23]. At controlled concentrations, these materials have shown to be non-toxic towards healthy mammalian cells [24-26]. With low toxicity, Cu and Cu oxide NPs can be easily applied in various medical applications/fields such as diagnostics, drug delivery and wound healing. Cu NPs are considered to be essential in the wound healing process due to their involvement in all stages of the wound healing process. Cu NPs have the ability to modulate various growth factors during wound healing. Various studies have shown the ability of Cu NPs to promote angiogenesis through regulation of the secretion of vascular endothelial growth factor [27-31].

In this study, dopamine capped Cu, Cu oxide and the mixture of Cu and Cu oxide (Cu/Cu oxide) NPs were synthesized through the chemical reduction method using hydrazine hydrate as a reducing agent. All experiments were carried out in ambient conditions. During Cu NPs synthesis, pure Cu was obtained without any oxide due to the presence of dopamine hydrochloride. Dopamine was also able to reduce Cu(II) to Cu(I) as observed during Cu oxide synthesis that was conducted without reducing agent. This is because Cu₂O was obtained instead of CuO and these observations were supported by PXRD and XPS data. The mixture of Cu and Cu₂O (Cu/Cu₂O) NPs were obtained when both hydrazine and dopamine hydrochloride were used following pre-treatment of Cu salt with ammonium hydroxide. The synergistic effect of Cu/Cu₂O NPs on the antimicrobial activity was investigated against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) bacterial strains. Separately, Cu and CuO NPs were found to be antimicrobial active; however, Cu/Cu₂O NPs demonstrated enhanced activity against both strains due to the NP synergistic effects. To investigate their wound healing potential, cytotoxicity studies of these NPs were carried out against baby hamster kidney fibroblasts (BHK-21) cell line.

5.2 Experimental

5.2.1 Materials

Copper (II) Chloride (CuCl₂, 99.9%), ammonium hydroxide (NH₄OH, 25%) hydrazine hydrate (NH₂NH₂.H₂O, 24-26%), dopamine hydrochloride, Trisodium citrate (99.0%), **Luria Bertani Broth** (LB), Tryptic Soy Agar (TSA), dimethylformamide (DMF, 99.99%), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were all

purchased from Sigma Aldrich and used as received. All glassware was washed with aquaregia and rinsed with distilled water.

5.2.2 Preparation of nanoparticles

To synthesize Cu NPs, 3% (v/v) of hydrazine hydrate was prepared in 100 mL distilled water. The solution was heated to boil in air. To the boiling solution, 10 mL of dopamine hydrochloride (1%, w/v) was added while stirring. After 10 minutes, 10 mL of CuCl₂ (1%, w/v) was added to the mixture. The reaction was carried out for 30 min while stirring. The product was cooled and washed with distilled water prior to characterization. Cu₂O NPs were prepared by adding 1 mL of NH₄OH in 100 mL of CuCl₂ (1% w/v). The solution was heated to boil, followed by the addition of dopamine hydrochloride (1% w/v). The reaction was carried out for 1 h with vigorous stirring. The product was cooled and washed with distilled water prior to characterization. Synthesis of Cu/Cu₂O NPs was achieved by heating the mixture of 1% (w/v) of CuCl₂ (in 100 mL) and 1 mL of NH₄OH to boil. To the mixture, 10 mL of dopamine hydrochloride (1% w/v) was added followed by the addition of 2 mL of hydrazine hydrate (1% v/v). The reaction was carried for 30 min, after which the product was cooled and washed for characterization.

5.2.3 Antimicrobial activity tests

5.2.3.1 Well diffusion method

Please refer to section 3.2.4.1 for detailed procedure.

5.2.3.2 Minimum inhibitory concentration (MIC) and bacteriostatic studies

Please refer to section 3.2.4.2 for detailed procedure. The final concentrations of the materials were ranging between 1.25 and 40 µg/mL.

5.2.4 Cell viability studies

Please refer to section 3.2.3 for detailed procedure. The final concentrations of the materials on the plates were ranging between 3.9 – 500 µg/mL.

5.2.5 Characterization techniques

All excess impurities from colloidal solutions were removed using a universal 320R centrifuge at a maximum speed of 15000 rpm. The absorption spectra were acquired using a Varian Cary Eclipse (Cary 50) UV-vis spectrophotometer. Transmission electron microscopy (TEM) images were obtained from FEI Tecnai-T12 microscopy at an electron acceleration

voltage of 120 kV and beam spot size of 10-100 nm. PXRD analysis was performed on a Bruker D2 Phaser with Cu. X-ray photoelectron spectroscopy (XPS) analysis was performed on a Physical Electronics PHI 5700 spectrometer. Non-monochromatic MgK α X-rays (300 W, 15 kV, and 1253.6 eV) were used as the excitation source. The spectrometer energy scale was calibrated using Cu 2p $_{3/2}$, Ag 3d $_{5/2}$, and Au 4f $_{7/2}$ photoelectron lines at 932.7, 368.3, and 84.0 eV, respectively. Samples were mounted on a sample holder without adhesive tape and kept overnight under high vacuum in the preparation chamber before being transferred to the analysis chamber for testing. The spectra were collected in the constant pass energy mode at 29.35 eV. The residual pressure in the analysis chamber was maintained below 1.33×10^{-7} Pa during the acquisition of the spectra using a multichannel hemi-spherical electron analyser.

5.3 Results and discussion

5.3.1 Characterization of the materials

Cu NPs were successfully synthesized in air with the aid of DA and hydrazine hydrate as capping and reducing agents, respectively. DA capped Cu₂O and Cu/Cu₂O NPs were obtained by first forming a Cu-ammonia complex followed by partial reduction aided by heating and addition of DA. The formation of the Cu-ammonia complex was noticed by change in colour of aqueous CuCl₂ solution from light to dark blue. The change in colour from dark blue to black after adding DA and heating was the confirmation of Cu₂O formation. Unlike with Cu₂O, the colours of Cu NPs and Cu/Cu₂O colloids were respectively found to be purple and maroon which indicated the formation or presence of zerovalent Cu. A digital picture depicting the colours of colloidal solutions of Cu, Cu₂O and Cu/Cu₂O NPs is shown in **Figure 5.1A**. Shown in **Figure 5.1B** are the UV-vis absorption spectra used to study the optical properties of the materials. Cu NPs spectrum (black) showed one pronounce characteristic absorption band at 587 nm. This absorption band was due to a surface plasmon resonance (SPR) phenomenon caused by coherence oscillation of conduction electrons on the surface of Cu NPs. The presence of this absorption band is a confirmation of successful synthesis of Cu NPs. A strong absorption band at 280 nm was observed from Cu₂O NPs spectrum (red). The presence of this absorption band is attributed to the existence of Cu₂O (cuprous oxide) instead of CuO (cupric oxide) phase of Cu oxide [32]. Two absorption bands at 293 nm and 605 nm were observed from the Cu/Cu₂O NPs spectrum (green). These absorption bands are respectively attributed to the presence and coexistence of Cu₂O and Cu NPs. The two absorption bands were red shifted and slightly broad compared to their

respective absorption bands. The red shift and the broadness of the absorption bands can be attributed to either agglomeration or bigger NPs size.

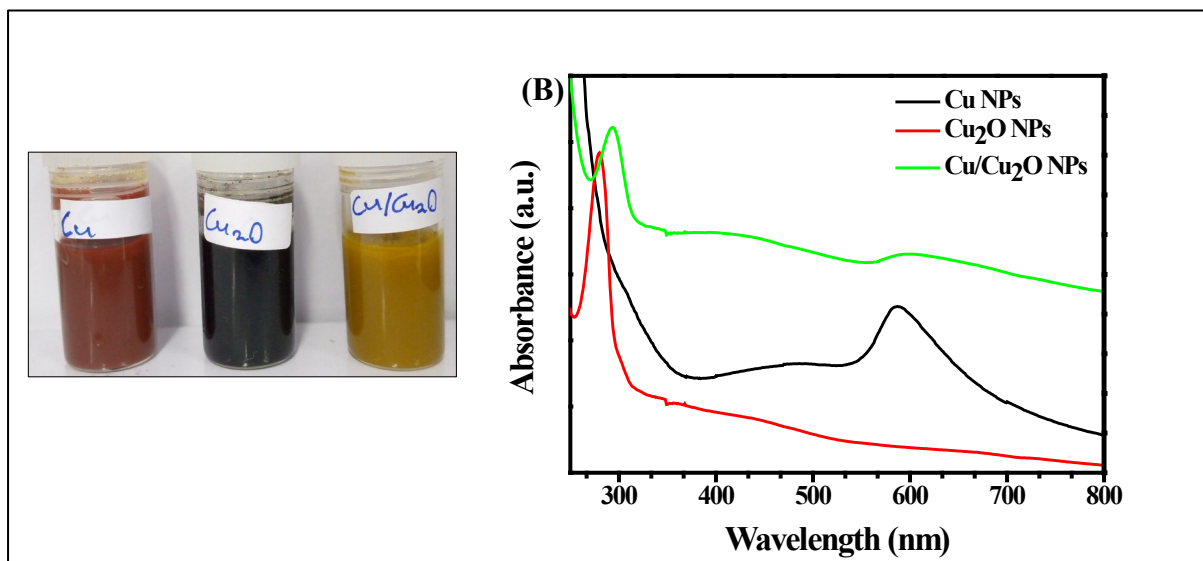


Figure 5.1: (A) A digital picture showing the colours of the colloidal solutions of (from left to right) Cu, Cu₂O and Cu/Cu₂O NPs; and (B) the UV-vis absorption spectra of Cu (black), Cu₂O (red) and Cu/Cu₂O NPs.

The phase purity and the crystallinity of Cu, Cu₂O and Cu/Cu₂O NPs were examined by PXRD as shown in **Figure 5.2**. The PXRD pattern of Cu NPs showed the characteristic diffraction peaks at 44°, 52° and 76° 2θ angles, corresponding to (111), (200) and (220) lattice planes. There were no other peaks observed which indicates that pure metallic CuNPs with the face centred cubic (FCC) crystal structure was successfully synthesized. This further illustrate that the presence of DA was able prevent the oxidation of CuNPs. The diffraction pattern perfectly matching to Cu₂O NPs phase was also obtained with all the characteristic diffraction peaks at 30°, 36°, 43°, 63° and 74° 2θ angles. These diffraction peaks correspond to (110), (111), (200), (220), and (311) lattice planes of Cu₂O (Cu⁺) with the FCC crystallite structure; further supporting the UV-vis observations. The diffraction pattern of Cu/Cu₂O NPs showed the characteristics diffraction peaks that correspond to the individual diffraction patterns of both the Cu and Cu₂O NPs. It's worth noting that the diffraction peak on the Cu/Cu₂O NPs pattern at 74° 2θ angle is the overlap of the Cu and Cu₂O NPs diffraction peaks (200) and (311). The formation of the Cu/Cu₂O NPs mixture further confirms that DA was able to prevent oxidation during the synthesis of pure Cu NPs. It can clearly be observed that the crystallite size of Cu NPs is big compared to that of Cu₂O due to the narrow and sharp diffraction peaks evident from the Cu/Cu₂O NPs diffraction patterns.

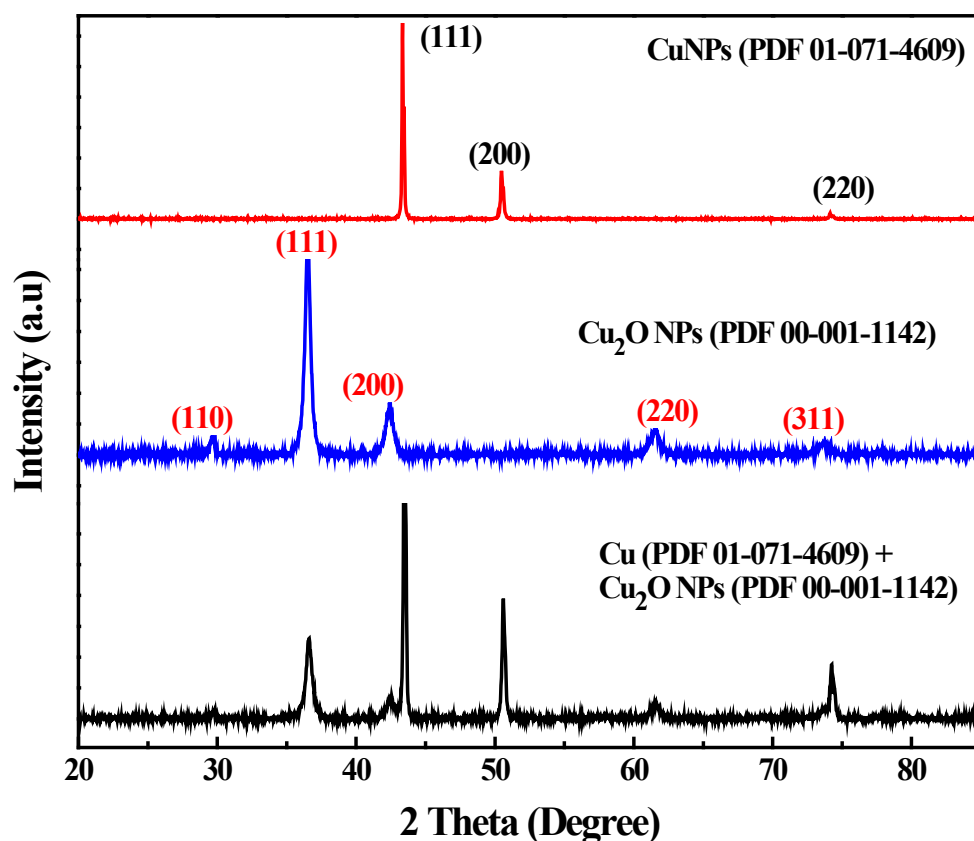


Figure 5.2: The PXRD patterns of Cu/Cu₂O (black), Cu₂O (blue) and Cu NPs (red).

The sizes and morphological characteristics of Cu, Cu₂O and Cu/Cu₂O NPs were studied using TEM analysis. **Figure 5.3**(A, B, C and D) shows the TEM micrographs of Cu, Cu₂O, Cu/Cu₂O and magnified Cu/Cu₂O, respectively. The size distribution histograms are shown in figure S1. The Cu NPs were found to have a spherical-like morphology and wide range size distribution with an average diameter of 14.7 nm as shown in **Figure 5.3** (A) and S1. Cu₂O were also found to have spherical morphology and narrow range size distribution with an average diameter of 6.3 nm as shown in **Figure 5.3** (B) and S5.1. The narrow range size distribution of Cu₂O can be attributed to the initial formation of Cu-ammonia complex $\{[\text{Cu}(\text{NH}_3)_4(\text{H}_2\text{O})_2]^{2+}\}$ prior to the addition of DA and heating, making it easy to control the size by preventing rapid growth and agglomeration. **Figure 5.3** (C and D) shows the TEM and magnified TEM micrographs of Cu/Cu₂O. Two different species of NPs with different sizes were observed. Both species have spherical-like morphologies, with the small NPs surrounding the big ones. The average diameters of both big and small NPs were found to be 92.0 nm and 10.6 nm, respectively. By the observations made from PXRD analysis, the small NPs represent Cu₂O NPs, and the big ones are Cu NPs. This was confirmed by broad and

sharp diffraction peaks for Cu_2O and Cu NPs, respectively from the PXRD pattern of Cu/ Cu_2O NPs.

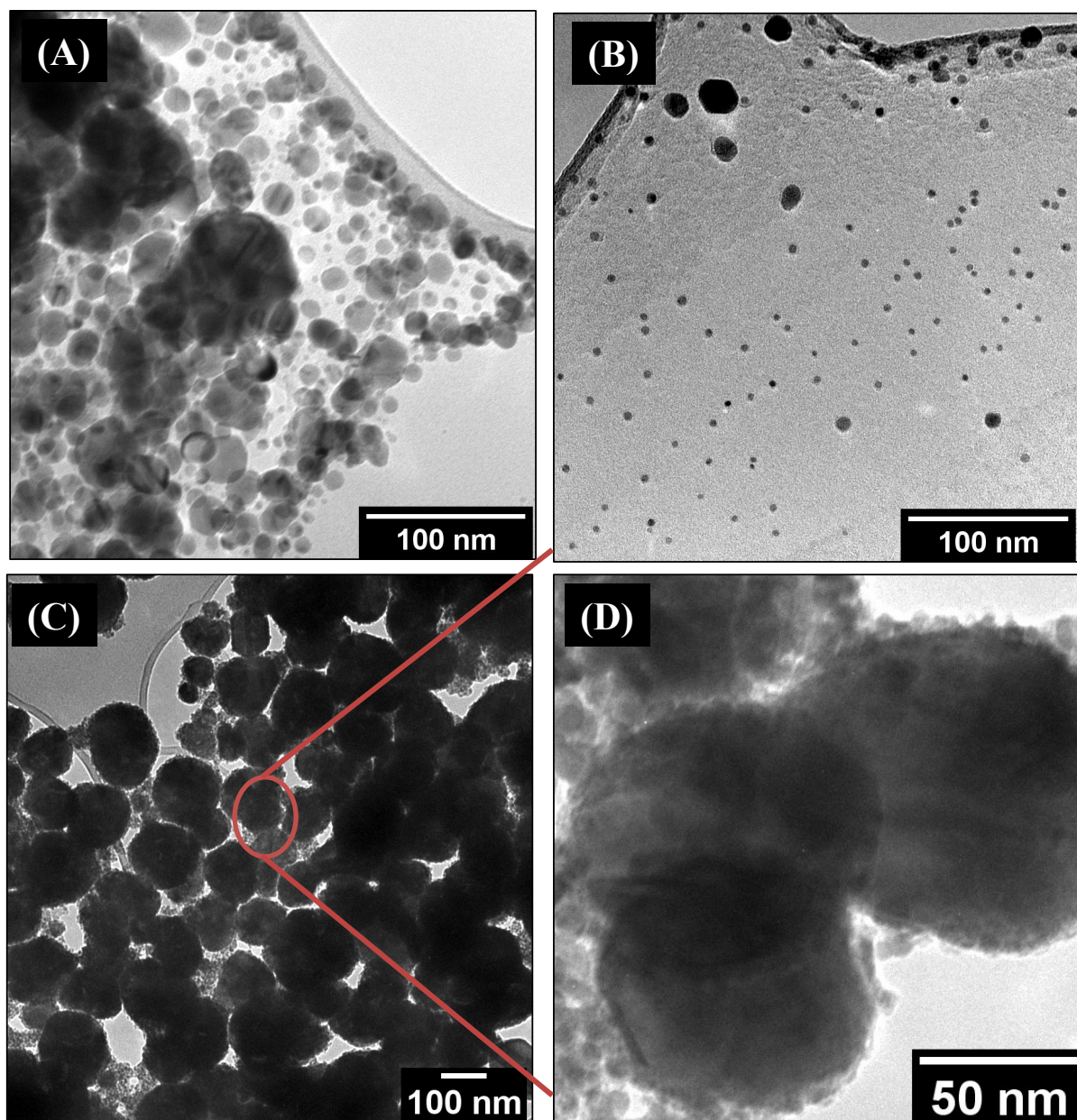


Figure 5.3: TEM micrographs of (A) CuNPs, (B) CuO, (C) Cu/CuO NPs and (D) magnified Cu/CuO.

The surface analysis, including chemical composition and the oxidation states of the NPs was carried out using XPS. The XPS survey spectra of Cu, Cu_2O and Cu/ Cu_2O NPs are shown in **Figure S5.2** (A, B and C). The survey spectra of the NPs showed the presence of O, N and C inherent from DA. **Figure 5.4** (A, C and E) and (B, D and F) respectively show the $\text{Cu}2p$ and $\text{O}1s$ scans for Cu, Cu_2O and Cu/ Cu_2O NPs deconvoluted high resolution XPS spectra. The $\text{Cu}2p$ spectrum of Cu NPs in **Figure 5.4A** shows two peaks at higher and lower Binding

energies (BEs) corresponding to $\text{Cu}2p_{1/2}$ and $\text{Cu}2p_{3/2}$. The $\text{Cu}2p_{1/2}$ peak at 953.3 eV confirms the presence of either Cu^0 or Cu^+ species. The $\text{Cu}2p_{3/2}$ peak was deconvoluted into two peaks at the BEs of 934.7 eV and 932.4 eV which correspond to Cu^{2+} and either Cu^+ or Cu^0 respectively. The Cu^{2+} peak was attributed to the presence of CuCl_2 and not CuO . This was confirmed by the $\text{Cl}2p$ spectrum in **Figure S5.2D** with the peak at 199.8 eV which correspond to CuCl_2 . Furthermore, the absence of satellite peaks from $\text{Cu}2p$ confirms that the Cu NPs sample only contain the Cu^0 species. The $\text{O}1s$ spectrum of Cu NPs in **Figure 5.4B** shows two oxygen species at BE of 533.2 eV and 531.7 eV. These peaks correspond to $\text{C}=\text{O}$ and $\text{C}-\text{O}$ groups which may be due to formation of DA quinone and DA, respectively. The presence of $\text{C}=\text{O}$ group shows that DA does play a role in reducing Cu ions by donating electrons through its acidic hydroxyl groups. This results into the oxidation of DA to form quinones that may bind or cap on the surface of Cu NPs. The absence of metal oxide peak further supports the observations made in $\text{Cu}2p$ spectrum and confirms the presence of only Cu^0 species in the Cu NPs sample.

Shown in **Figure 5.4(C and D)** are the $\text{Cu}2p$ and $\text{O}1s$ spectra of Cu_2O NPs. The two peaks at high and lower BEs from $\text{Cu}2p$ spectrum correspond to $\text{Cu}2p_{1/2}$ and $\text{Cu}2p_{3/2}$ respectively. Unlike in Cu NPs spectrum, the $\text{Cu}2p_{1/2}$ was deconvoluted in two peaks at the BEs 953.3 eV and 952.4 eV corresponding to Cu^{2+} and Cu^+ , respectively. The $\text{Cu}2p_{3/2}$ peak was also deconvoluted into two peaks at the BEs of 933.8 eV and 932.7 eV which also correspond to Cu^{2+} and Cu^+ . A weak satellite was also observed at the BE of 945.2 eV. A weak satellite peak is confirmation of Cu_2O presence instead of CuO and further advocates the reduction of Cu^{2+} to Cu^+ [33, 34]. CuO is characterized by a strong satellite peak as previously reported in literature [36]. The presence of Cu^{2+} in this sample can be attributed to surface oxidation during characterization. PXRD analysis also confirmed that this sample contain Cu_2O instead of CuO phase. XPS can detect CuO that forms an outer amorphous shell due to surface oxidation since it is able to give surface information of materials within the depth of 5 to 10 nm, whereas PXRD analyse the information of the bulk material. The $\text{O}1s$ spectrum of Cu_2O NPs shows three deconvoluted peaks at the BE of 533.2 eV, 531.8 eV and 530.2 eV. The peak at lower BE (530.2 eV) is attributed to O^{2-} phase that interact with the Cu atoms to form Cu_2O , and the other two peaks at 533.2 eV and 531.8 eV are respectively attributed to the $\text{C}=\text{O}$ and $\text{C}-\text{O}$ species inherent from DA and DA quinones. The absorbed oxygen atoms peak may have overlapped with DA $\text{C}-\text{O}$ peak at around 531.8 eV.

Figure 5.4(E and F) show the Cu2p and O1s spectra of Cu/Cu₂O NPs with the characteristic Cu2p_{1/2} and Cu2p_{3/2} peaks at higher and lower BEs, respectively. Both Cu2p_{1/2} and Cu2p_{3/2} peaks were deconvoluted into two peaks each. The peaks at the BE of 955.4 eV (Cu2p_{1/2}) and 934.9 eV (Cu2p_{3/2}) respectively correspond to the presence of Cu²⁺ species. The presence of Cu⁺ and Cu⁰ species was collectively confirmed by the peaks at the BE of 952.5 eV (Cu2p_{1/2}) and 932.7 eV (Cu2p_{3/2}). The presence of a weak, instead of a strong shake-up satellite peak at 554.9 eV is a confirmation that the Cu oxide that is present in the sample is Cu₂O instead of CuO. Furthermore, the absence of a second strong satellite peak around 960 eV is a confirmation that the sample contains Cu₂O instead of CuO. The Cu²⁺ species observed from the spectrum was due to the surface oxidation of the materials. **Figure 5.4F** shows the O1s spectrum of Cu/Cu₂O NPs which is like that of Cu₂O. Three deconvoluted peaks at BE of 533.3 eV, 531.7 eV and 530.4 eV were also observed. These peaks correspond to C=O, C-O and Cu oxide. The presence of C=O group shows the presence of DA quinone due to oxidation of DA as in the Cu NPs spectrum. The Cu oxide peak at 530.4 eV is attributed to the formation of Cu₂O. The absorbed oxygen atoms peak may have overlapped with DA C-O peak at around 531.7 eV.

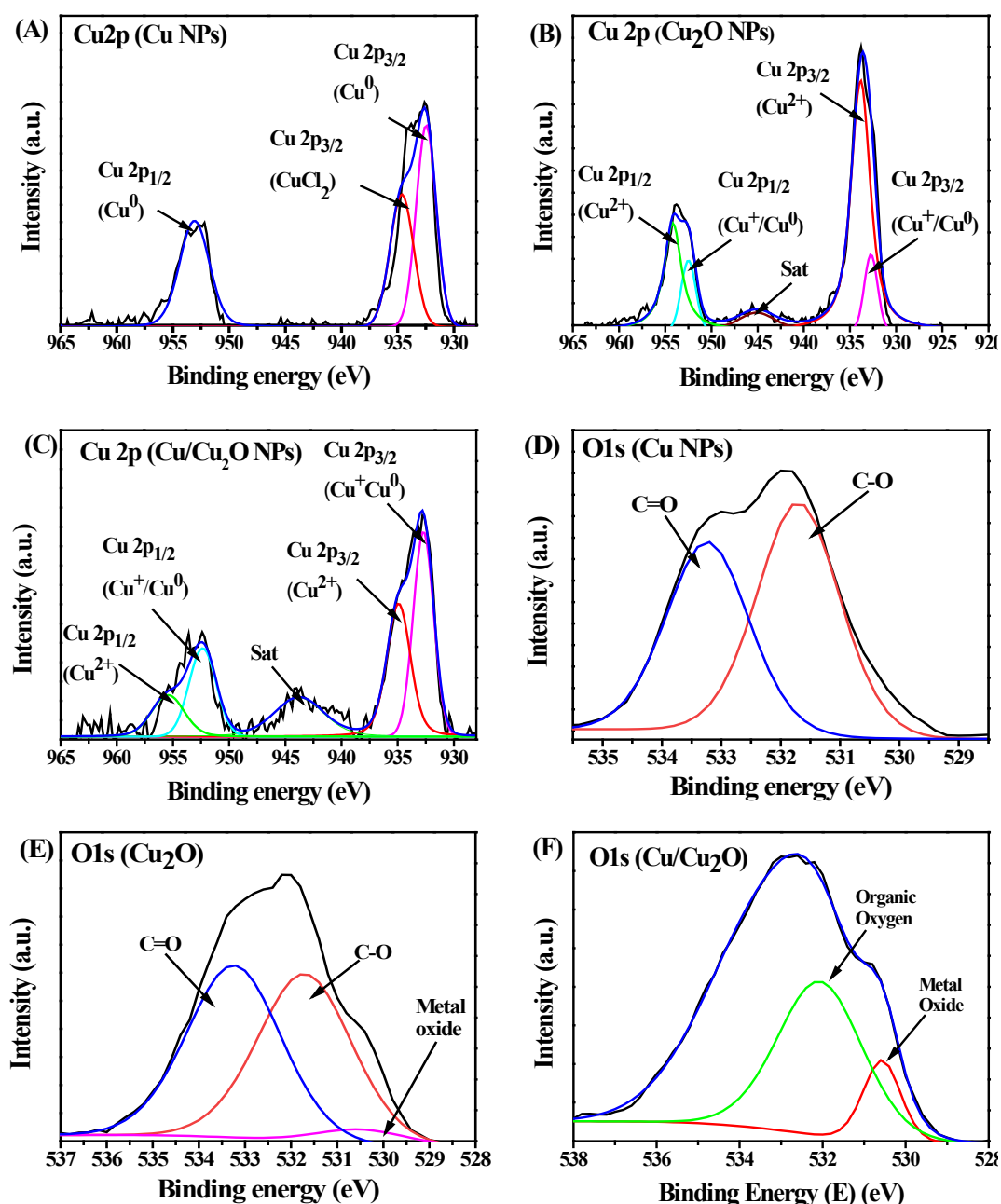


Figure 5.4: High resolution XPS spectra showing (A, C and E) O1s scans and (B, D and F) Cu2p scans of Cu, Cu₂O and Cu/Cu₂O NPs.

5.3.2 Toxicity studies of the synthesized materials

The cytotoxicity effect of the synthesized Cu, Cu₂O and Cu/Cu₂O NPs was tested using the MTS assay by assessing the viability of BHK-21 cells as shown in **Figure 5.5**. The cells were exposed to various concentrations of the NPs for 72 h. The cells treated with DMEM only, without NPs, were used as control. A cell viability of approximately 100 % was observed, indicating that the cells were not affected throughout the study. The NPs showed a certain

degree of toxicity at very low concentrations. At 3.9 $\mu\text{g/mL}$ almost 20% of the cells were nonviable. At least 80% of the BHK-21 cells treated with 3.9 $\mu\text{g/mL}$ of Cu/Cu₂O and CuNPs were still viable. A cell viability of 75% was observed for cells treated with Cu₂O NPs at the same concentration of 3.9 $\mu\text{g/mL}$. Cu₂O NPs appeared to have more negative impact on the cell viability compared to Cu and Cu/Cu₂O NPs. This was clearly indicated by a drastic decrease in cell viability as the concentration of Cu₂O NPs increased. At 15.6 $\mu\text{g/mL}$ of Cu₂O NPs, the cell viability decreased to less than 50%, while it was at approximately 60 and 50% for Cu/Cu₂O and CuNPs respectively. Cu/Cu₂O NPs maintained relatively good cell viability of about 60% at concentrations as high as 62.5 $\mu\text{g/mL}$. The cell viability dropped below 50% when the concentration of Cu₂O NPs increased to 15.6 $\mu\text{g/mL}$. In general, the toxicity of all the NPs was found to be concentration dependent. It is clear that Cu₂O possessed strong cytotoxic effect than both Cu and Cu/Cu₂O NPs at various concentrations. This could be due to the oxidative stress caused by the high release of reactive oxygen species (ROS) by Cu₂O compared to the other materials. **Figure S5.3** represents the sigmoid curves used to determine the 50% cytotoxic concentrations (CC₅₀) of Cu, Cu₂O and Cu/Cu₂O NPs tested against BHK-21 cells. The CC₅₀ values of these materials were determined to evaluate the applicable concentrations in biomedical applications. As shown in **Table 5.1**, the CC₅₀ for Cu/Cu₂O, Cu and Cu₂O NPs were found to be 50.1, 15.1 and 10.0 $\mu\text{g/mL}$ respectively. The CC₅₀ value of Cu/Cu₂O NPs is relatively high compared to those of Cu and Cu₂O NPs. This is an indication that Cu/Cu₂O NPs is less toxic towards BHK-21 cells compared to Cu and Cu₂O NPs.

The presence of DA can lead to bioconjugation that may reduce exposure, dissolution of Cu, and release of Cu ions from Cu and Cu/Cu₂O NPs leading to less toxicity towards the cells, however it was not the case with Cu₂O. It has been reported that Cu ions are one of the main causes of toxicity of Cu based materials. The size of NPs also plays a huge role in their toxicity. The other main reason Cu₂O was more toxic than the other materials could be due to their small particles sizes as observed from TEM and PXRD analysis. Small NPs can easily penetrate cell membranes and interact with vital organs, disturbing normal physiological behaviour of the cells.

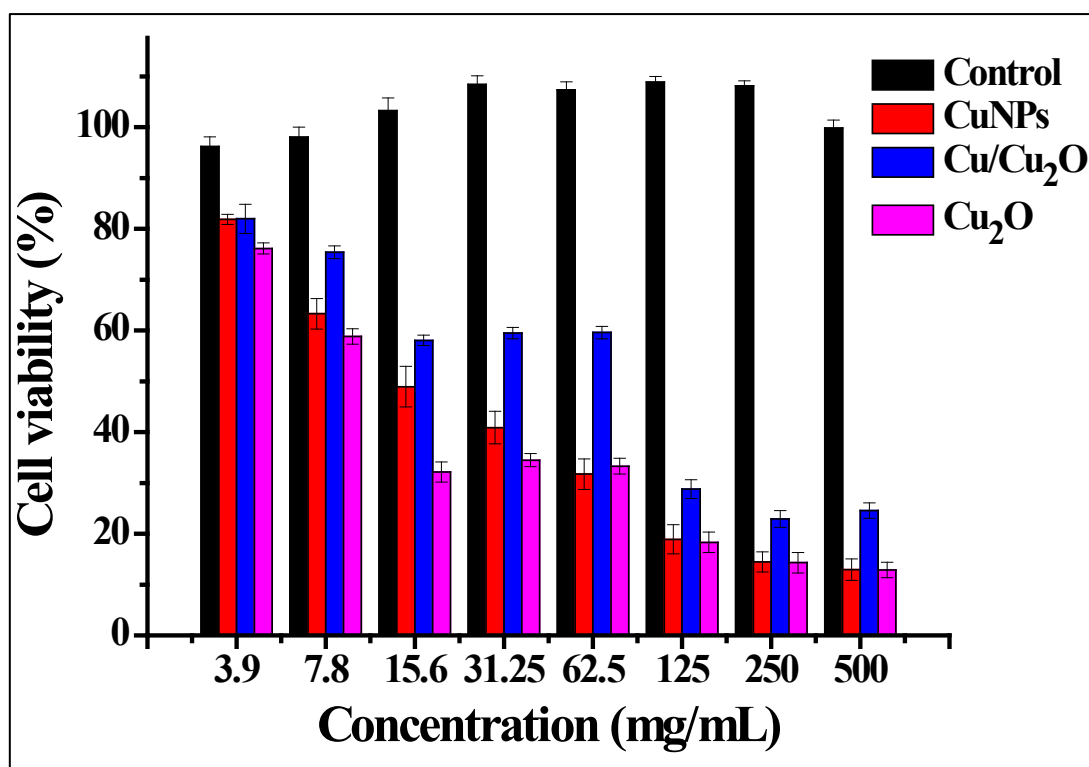


Figure 5.5: Cell viability of BHK-21 cells exposed to Cu, Cu₂O and Cu/Cu₂O NPs at different concentrations.

5.3.3 Antimicrobial activity of the materials

The antimicrobial activity of Cu, Cu₂O and Cu/Cu₂O NPs was evaluated using a standard well-diffusion method against *E. coli* and *S. aureus*. Shown in **Figure 5.6** are the digital images of the agar plates with clear inhibition zones around the wells. The inhibition zones around the wells are an indication of the antimicrobial effect of the NPs. The diameters of the inhibition zones were measured with a ruler and recorded in **Table 5.1**. All the synthesized NPs exhibited a certain level of antimicrobial activity against *E. coli* and *S. aureus*. This antimicrobial study was conducted using a concentration of 10 µg/mL, which equates to the CC₅₀ value of the most toxic NPs (Cu₂O NPs). Both Cu and Cu₂O NPs showed a relatively low antimicrobial activity against both *E. coli* and *S. aureus* compared to Cu/Cu₂O NPs. This is shown by big inhibition zones on the agar plates. Inhibition zones of approximately 40 mm were observed when Cu/Cu₂O NPs were tested, while Cu and Cu₂O NPs resulted in the formation of inhibition zones with diameters less than 10 mm. The extraordinary antimicrobial activity of Cu/Cu₂O NPs can be attributed to their synergistic effect as a result of the independent mechanism of action possessed by each material (either Cu or Cu₂O). The antimicrobial properties of these NPs were further evaluated by determining their minimum

inhibitory concentration (MIC) values. The determined MICs for Cu, Cu₂O and Cu/Cu₂O NPs are summarized in **Table 5.1**. The MIC values for Cu and Cu₂O NPs were found to be 10 µg/mL against both *E. coli* and *S. aureus*. A very low MIC value of 1.25 µg/mL was observed for Cu/Cu₂O NPs against both *E. coli* and *S. aureus*. This shows that the concentration of Cu/Cu₂O NPs required to inhibit bacterial growth is 8 times less than that required for Cu and Cu₂O NPs. The low MIC of Cu/Cu₂O NPs further proves their synergistic effect towards bacterial growth inhibition. These SI values of the materials were determined to investigate their toxicity/efficacy relationship. As shown in **Table 5.1**, the SI values obtained for the Cu, Cu₂O and Cu/Cu₂O NPs were 1.5, 1 and 76.1, respectively, against both *E. coli* and *S. aureus*. These results show the Cu₂O NPs to be the least safe, followed by the Cu NPs, with the Cu/Cu₂O NPs being the only ones that presented favourable safety margins.

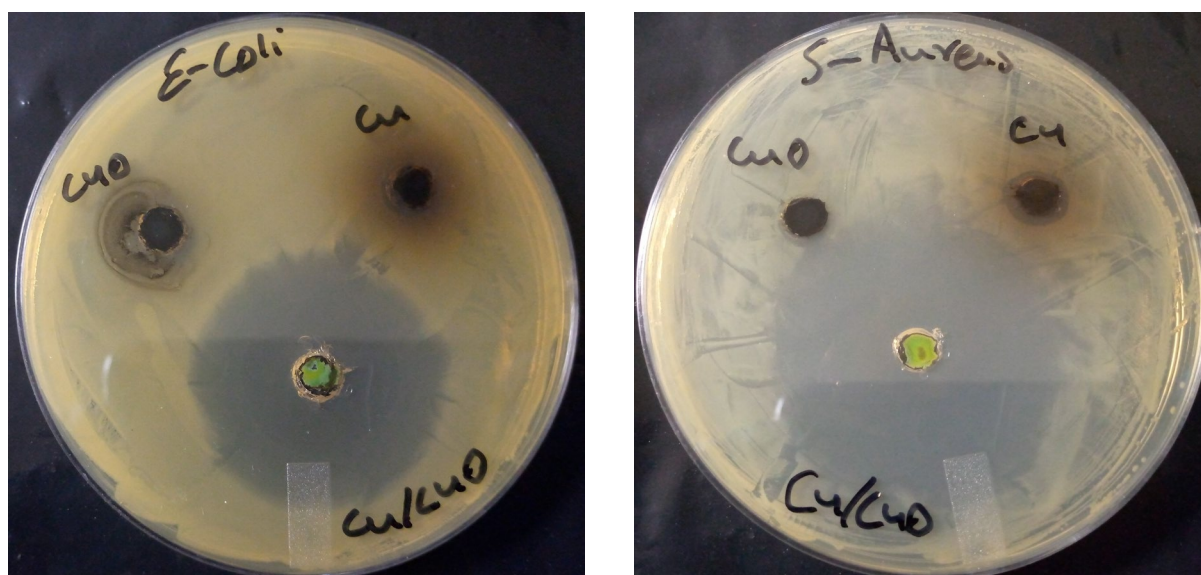


Figure 5.6: Agar plates showing zones of inhibition due to Cu₂O NPs, CuNPs and Cu/Cu₂O NPs against *E. coli* and *S. aureus*.

Table 5.1: Summary of the inhibition zones, MIC, CC₅₀ and SI values of the synthesized NPs

Sample	Zone of Inhibition (mm)		MIC ($\mu\text{g/mL}$)		CC ₅₀ ($\mu\text{g/mL}$)	SI value	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>		<i>E. coli</i>	<i>S. aureus</i>
Cu NPs	7	9	10	10	15.1	1.5	1.5
Cu ₂ O NPs	10	7	10	10	10.0	1.0	1.0
Cu/Cu ₂ O NPs	36	43	1.25	1.25	95.1	76.1	76.1

5.4 Conclusions

The study successfully showcased a simple synthetic route, characterization, cytotoxicity and antimicrobial activity of Cu and Cu oxide NPs. The UV-vis spectrometry was used to determine the optical properties and further assisted in distinguishing the nature of the NPs. Pure Cu NPs were characterized by a single characteristic absorption band at a lower energy region due to SPR. Cu oxide also showed one absorption band towards higher energy region. The absorption spectrum of Cu/Cu₂O NPs showed two absorption bands which confirmed the presence and coexistence of Cu (at lower energy region) and Cu oxide (at higher energy region). PXRD confirmed the crystallinity and phases of the as-synthesized materials. The FCC structures for both Cu and Cu oxide were obtained. The PXRD analysis further confirmed the Cu₂O instead of CuO was synthesized. The surface analysis of the materials was further investigated using XPS which supported the findings from PXRD analysis. The cytotoxicity profiles of the NPs were successfully investigated using BHK-21 cells. Cu₂O NPs appeared to be more toxic compared to both Cu and Cu/Cu₂O NPs. Of all the synthesized NP, Cu NPs were less toxic with the CC₅₀ at least 3 and 5 times higher than that of Cu/Cu₂O and Cu₂O NPs, respectively. An interesting antimicrobial effect of the NPs against *E. coli* and *S. aureus* bacterial strains was observed from this study. Cu, Cu₂O and Cu/Cu₂O NPs were all found to be able to inhibit bacterial growth, with the synergistic effect of the mixture of Cu and Cu oxide nanoparticles showing 8 times more potency in inhibiting bacterial growth than Cu and Cu₂O NPs. The MIC of Cu/Cu₂O NPs was at least 12 times lower than their CC₅₀, while MICs of Cu and Cu₂O NPs were 5 and 1 times their CC₅₀ values, respectively. Taken together, these results show that the Cu/Cu₂O NPs were much more potent and safer (lower MIC and higher SI value) compared to the Cu and Cu₂O NPs. However, since the CC₅₀ values obtained for these NPs were low, the cytotoxicity of these

materials would have to be evaluated against a wide range of mammalian cells to ensure their safety.

5.5 References

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Chapter 6

***In vitro* investigation of wound healing ability of copper-polydopamine nanocomposites**

6.1 Introduction

The prevalence of bacterial infections in wounds has posed a serious health problem around the world. Infected wounds can lead to several irreversible/permanent damages to human bodies, including but not limited to limb amputation and fatality [1-4]. Moreover, a wound can also act as a path for pathogens to enter and disrupt the body's immune system which may result in various infections and diseases [3]. During wound healing, the body produces vital cells that fight infection, remove wound debris and regenerate skin tissue. However, when the bacteria burden increases and the wound gets infected, the healing process gets prolonged. Therefore, a strong intervention is needed when bacteria start to pose a problem in wound healing to avoid deteriorations. The process of wound healing involves four complicated and overlapping physiological stages i.e., inflammation, proliferation, differentiation and cell migration. To achieve a normal recovery and anatomical functions of skin, development of excellent wound dressing materials is crucial. Recent studies have focused on developing advanced wound dressing materials that are able to modulate tissue regeneration, respond to physiological signals with good biological activities [5-8]. Advanced wound dressings based on hydrocolloids, hydrogels, films and nanocomposites have shown great potential in wound healing [9-12]. Nanocomposites have been widely used in wound healing because they combine the intrinsic properties of different materials for synergistic results. For instance, combining antimicrobial active nanoparticles and biodegradable polymers offers a wound healing system that will prevent bacterial infections at the same time controlling exudates and facilitating cell migration for fast skin tissue regeneration. The most prominent nanocomposites are either polymer or metal nanoparticles (NPs) incorporated on the surface or within the matrix of biodegradable polymers. Other material such as carbon, zeolite and silica have also been incorporated with metal NPs and polymers to produce nanocomposites with wound healing properties [13-15].

Metal NPs such as silver (Ag), gold (Au) and copper (Cu) have been incorporated with other material for wound healing nanocomposites [16-18]. These NPs are known for their wide spectrum of antimicrobial activity. They have also been reported to play a huge role in various stages of the wound healing process. Ag and Au NPs are known to have the ability to activate the secretion of various growth factors and cytokines during the inflammatory stage. Cu NPs on the other hand can play a role of collagen synthesis and fibroblasts proliferation during the proliferative stage [19]. Besides having wound healing properties, Cu is an essential metal that plays vital roles in the human body. It facilitates the development and maintenance of cardiovascular, skeletal, and nervous systems [20]. Therefore, the leaching of Cu NPs inside the body during wound healing treatment is not a problem if the concentration is controlled.

Cu NPs have been incorporated with various biodegradable polymers for improved wound healing purposes. Synthetic and natural polymers [21-23] have widely been used in wound healing application. These polymers are biocompatible, biodegradable, non-toxic and some of them have antimicrobial properties. Polymers play a huge role in wound healing such as collagen synthesis, angiogenesis, re-epithelialization and extracellular matrix ((ECM) remodelling [24]. A study by Gopal *et al.* revealed a pro-angiogenic capacity of chitosan incorporated with Cu NPs nanocomposites [25]. This nanocomposite was able to activate different cytokines and growth factors with promising results in the treatment of cutaneous wounds. In another study, Cu NPs loaded on phenytoin were able to stimulate granulation and tissue formation, which further accelerated the epidermal regeneration in a wounded rat [26].

Polydopamine (PDA) is one of the synthetic polymers that have been widely used in the wound healing application. A hydrogel made of PDA incorporated with puerarin and polyethylene glycol diacrylate was found to possess excellent wound healing properties with antioxidant activity [27]. A study by Zhang *et.al.* showed that coating silk fibroin membranes with PDA modified their surface properties and further improved cell attachment and fibroblasts proliferation. The *in vivo* study on rat skin showed an accelerated wound closure due to PDA modified silk fibroin [28]. O'Connor *et. al.* showcased the antioxidant activity of PDA incorporated with polyethylene imine and dextran. This hydrogel-based composite was found to influence cell migration in an *ex vivo* wound healing assay [29]. A few studies have demonstrated the incorporation of Cu NPs on PDA, and to the best of our knowledge, none have applied them in wound healing all together [30-36]. Therefore, this study showcased the

fabrication of antimicrobial active PDA/Cu NPs nanocomposites and their potential in wound healing. Cu NPs were either supported on the surface or embedded within the matrix of PDA. The effect of Cu ion concentration on the NPs dispersion was investigated. These nanocomposites were found to be biocompatible with low toxicity towards baby hamster kidney fibroblast (BHK-21) cells at monitored concentrations.

6.2 Experimental

6.2.1 Materials

Copper chloride (CuCl_2 , 99.9%), dopamine hydrochloride, Tryptic Soy Agar (TSA), dimethylformamide (DMF, 99.99%), penicillin-streptomycin, Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were all purchased from MERCK and used as received. All glassware were washed with aqua-regia and rinsed with distilled water.

6.2.2 Synthesis of nanocomposites

6.2.2.1 Preparation of CuNPs on the surface of PDA (Cu@PDA)

PDA spheres were prepared by mixing 50 mL distilled water and 25 mL ethanol. To the mixture, 1 mL ammonium hydroxide (30-33%) was added and the reaction was gently stirred for 30 minutes. Dopamine hydrochloride (0.1 g) was dissolved in distilled water and added to the mixture. The reaction was carried out for 30 hours and the product was washed with distilled water with the aid of a centrifuge at 15000 rpm for 15 minutes and dried at room temperature before characterization. To incorporate CuNPs, 50 mg of PDA was dispersed in 50 mL distilled water using an ultrasonicator. Various amounts of CuCl_2 (5, 10 and 50 mg) were added (10 mL) to the PDA suspension followed by 10 mL of 1% hydrazine while stirring. The reaction was carried out for 2 h at room temperature. The product was washed 2 times with distilled water using centrifuge and dried for characterization.

6.2.2.2 Preparation of CuNPs in PDA matrix (Cu-PDA)

To synthesize CuNPs embedded in PDA matrix, 0.1 g of CuCl_2 and 0.1 g of dopamine hydrochloride were dissolved in 100 mL distilled water followed by 1 mL of ammonium hydroxide (30-33%). After 24 h of stirring at room temperature, 1 mL of hydrazine hydrate (78-80 %) was added. The reaction was further carried out for another 30 min while stirring. The reaction was stopped, and the product was washed with distilled water with the aid of centrifuge.

6.2.3 Cell viability studies

Please refer to section 3.2.3 for detailed procedure. The final concentrations of the materials were ranging between 7.8 – 1000 µg/mL.

6.2.4 Antimicrobial activity tests

6.2.4.1 Well diffusion method

Please refer to section 3.2.4.1 for detailed procedure.

6.2.4.2 Minimum inhibitory concentration (MIC) and bacteriostatic studies

Please refer to section 3.2.4.2 for detailed procedure. The concentration range of the nanocomposites was 100 to 0.78125 µg/mL.

6.2.5 Characterization

All excess impurities from colloidal solutions were removed using a universal 320R centrifuge at a maximum speed of 15000 rpm for 15 minutes. Chemical structure analysis was done using a Bruker Tensor 27 Fourier transform infrared (FTIR) spectrometer. Thermal analysis was conducted using a Perkin Elmer TGA 6000 thermogravimetric analyser (TGA) using high-purity nitrogen at a heating rate of 10 °C/min and gas flow rate of 10 mL/min. Transmission electron microscopy (TEM) images were obtained from FEI Tecnai-T12 microscopy at an electron acceleration voltage of 120 kV and beam spot size of 10-100 nm. The TEM particle diameters were determined using ImageJ software. Powdered X-ray diffraction (PXRD) analysis was performed on a Bruker D2 Phaser with Cu radiation and each scan was recorded over 20 min.

6.3 Results and discussion

6.3.1 Materials characterization

The surface morphology and the particles sizes of the nanocomposites were studied using TEM and SEM. Shown in **Figure 6.1** is the TEM micrographs of the as-synthesized nanocomposites. **Figure 6.1(a-c)** shows the micrographs of CuNPs embedded in the polydopamine matrix to form Cu-PDA nanocomposite at various concentrations of Cu salt (7.4, 3.7 and 0.74 mM). The sizes of CuNPs within the PDA matrix appeared to be concentration dependent. At high concentration of Cu salt (**Figure 6.1a**), the nanoparticles with big sizes were obtained. The sizes of the nanoparticles decreased with decreasing concentration of Cu salt (**Figure 6.1b and c**). Particles sizes were determined by obtaining the average diameter using imagej software, and their particles size distributions are presented in **Figure S6.1 (a-c)**. The average diameters of CuNPs were found to be 65, 32 and 19 nm for Cu-PDA prepared by Cu salt concentration of 7.4, 3.7 and 0.74 mM respectively. At high concentration of Cu ions, more nuclei are formed followed by the growth process resulting into bigger particles due to Ostwald ripening. Even though the nanoparticles grow bigger with increasing concentration of Cu salt, there was no evidence of agglomeration. This shows that the PDA matrix was able to protect the nanoparticles from agglomeration.

Figure 6.1 (d-f) shows the TEM micrographs of CuNPs supported on the surface of PDA spheres to form Cu@PDA nanocomposites. The effect of Cu salt concentration on the dispersion and sizes of CuNPs was also investigated as in the case of Cu-PDA nanocomposites. CuNPs with big sizes were obtained following the use of high concentration of Cu salt (7.4 mM) as indicated in **Figure 6.1d**. As the concentrations of the Cu salt decrease to 3.7 and 0.7 mM, the sizes of CuNPs on the surface of PDA spheres respectively decreased to 45 and 9 nm as shown in **Figure 6.1 (e and f)**. These observations were also made from Cu-PDA samples. However, at high concentrations of Cu salt (7.4 and 3.7 mM), agglomeration of CuNPs was observed. The agglomeration may be due to the uncontrolled growth of nanoparticles since they are exposed to each other on the surface of PDA spheres, unlike in Cu-PDA nanocomposites where nanoparticles are within the matrix.

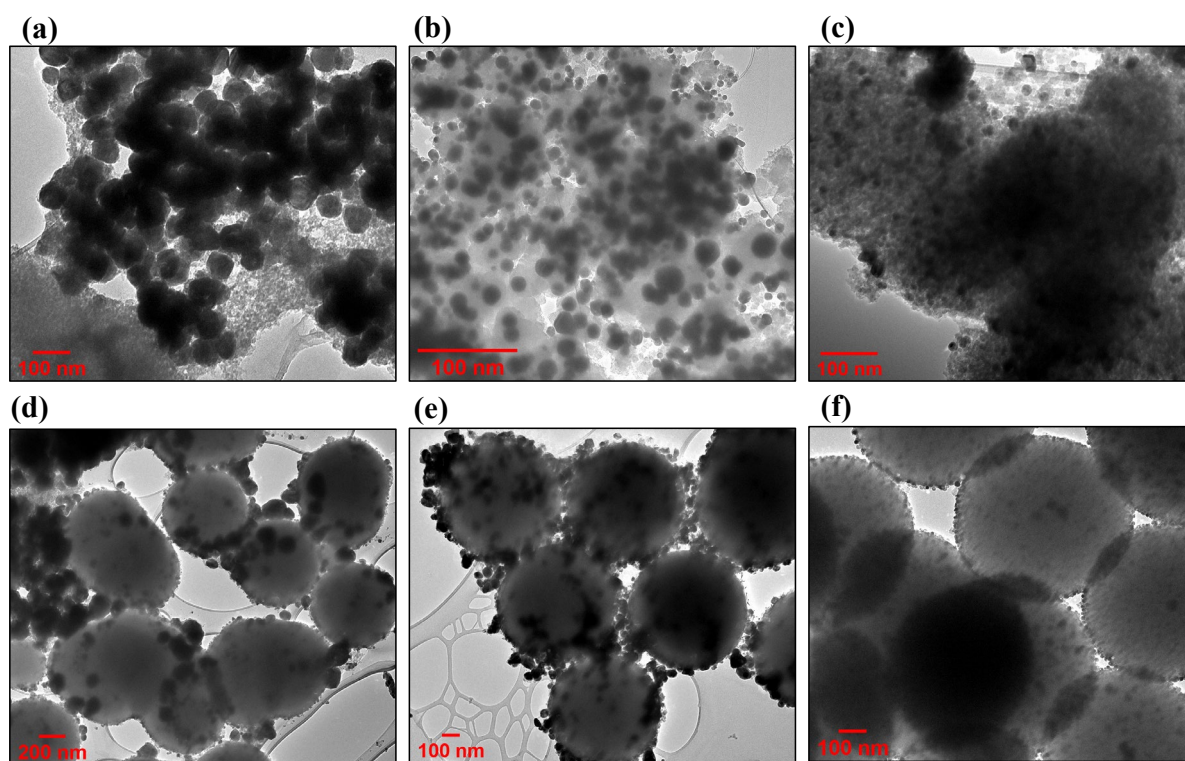


Figure 6.1: TEM images of (a-c) Cu-PDA and (d-f) Cu@PDA nanocomposites at different CuCl_2 concentrations. [(a, d) 7.4 mM, (b, e) 3.7 mM and (c, f) 0.74 mM].

The surface morphologies of the nanocomposites were further studied by SEM analysis. Shown in **Figure 6.2** are the SEM micrographs of (a-c) Ag-PDA and (d-f) Cu@PDA nanocomposite. Cu-PDA is characterized by the irregular morphology as shown in **Figure 6.2** (a-c), which is a representation of the PDA embedding CuNPs within the matrix structures. **Figure 6.2** (d-f) shows the Cu@PDA nanocomposites with a clear indication of CuNPs supported on the PDA spheres. The PDA spheres appeared to have rough surfaces with spherical morphology. The agglomerates of CuNPs on PDA spheres were observed in **Figure 6.2** (d and e) as a result of high concentrations of Cu salt used. These observations are in support with TEM analysis.

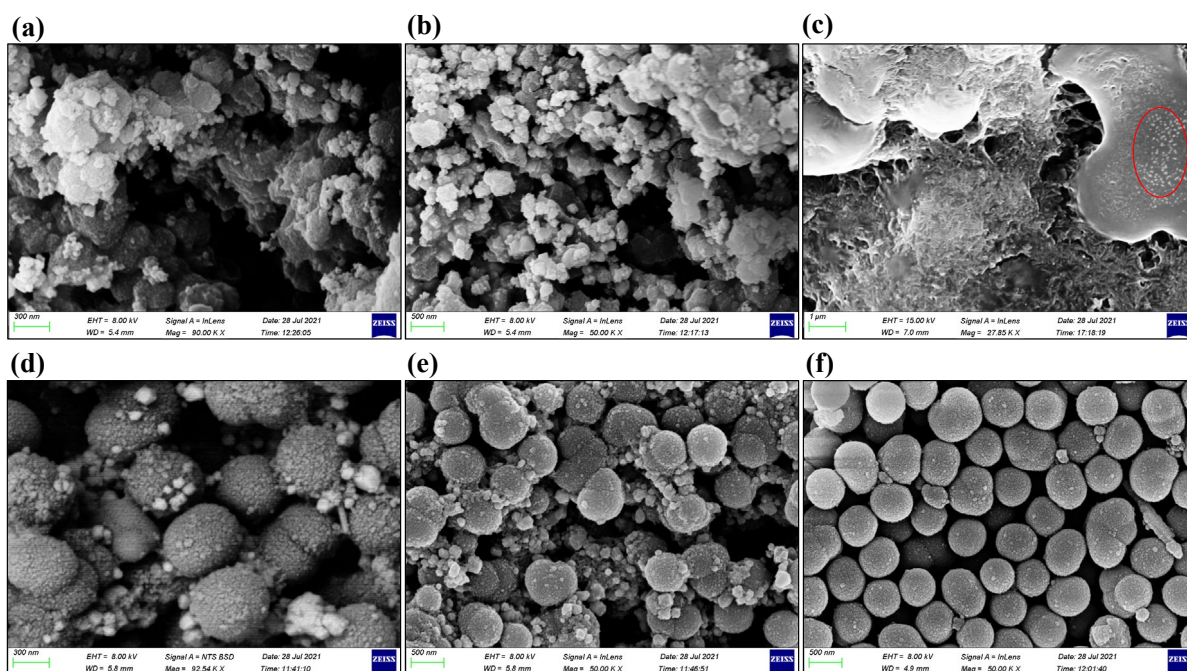


Figure 6.2: SEM images of (a-c) Cu-PDA and (d-f) Cu@PDA nanocomposites at different CuCl_2 concentrations. [(a, d) 7.4 mM, (b, e) 3.7 mM and (c, f) 0.74 mM].

The crystal structures and crystallinity of Cu-PDA and Cu@PDA nanocomposites were investigated using PXRD. Shown in **Figure 6.3** (a and b) are the diffractograms of Cu-PDA and Cu@PDA prepared with (i) 7.3 mM, (ii) 3.7 mM and (iii) 0.7 mM. The diffractograms of both Cu-PDA and Cu@PDA were characterized by three diffraction peaks around 43° , 50° and 74° diffraction angles which correspond to (111), (200) and (220) crystal lattice planes of face centred cubic phase of metallic Cu (PDF: 01-071-4609). There was no evidence of other peaks which may be due to the presence of Cu oxide or other impurities. Relatively sharp diffraction peaks for Cu-PDA (i) and Cu@PDA (i and ii) were observed. The sharp diffraction peaks are due to big crystallite or grain size of the particles. These observations can be related to the big particles average diameters observed from TEM analysis for Cu-PDA and Cu@PDA prepared at high concentrations of Cu salts. The diffraction peaks started to broaden as the concentration of the Cu salt decreases indicating small crystallite or grain size of the particles. It is also important to note small humps from some of the diffractograms. These humps were attributed to the amorphous peaks due to the presence of PDA.

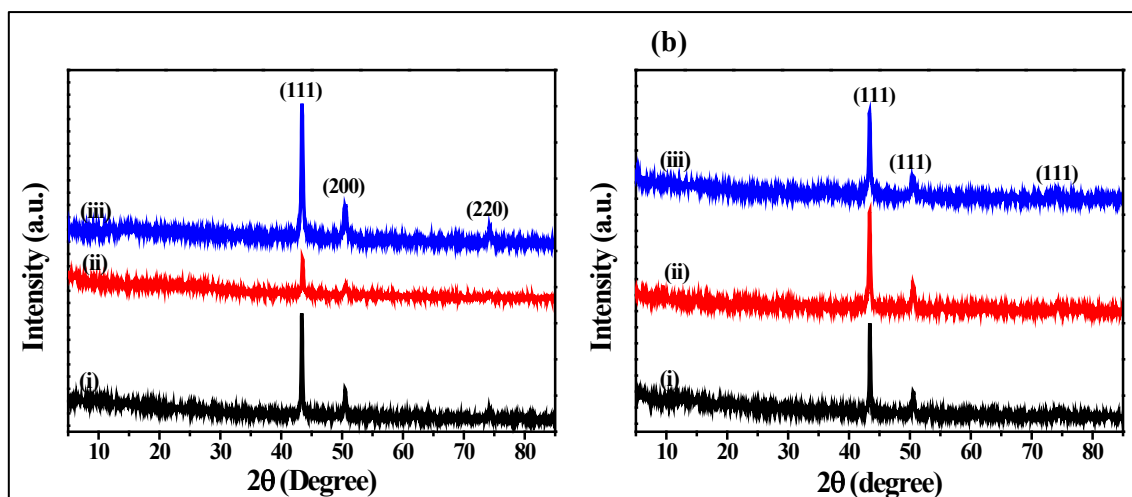


Figure 6.3: PXRD patterns of (a) Cu-PDA and (b) Cu@PDA nanocomposites at different CuCl₂ concentrations. [(i) 7.4 mM, (ii) 3.7 mM and (iii) 0.74 mM].

FT-IR spectroscopic technique was utilized for structural analysis of Cu-PDA and Cu@PDA nanocomposites as shown in **Figure 6.4** (a and b). For clarity purpose, the FT-IR analysis of PDA was done and used a reference for the nanocomposites analysis. The FT-IR spectra in figure 4a(i) and b(i) showed well-defined characteristics functional groups of PDA. The characteristic vibrational bands around 3300 cm^{-1} which correspond to the overlapping of -OH and -NH were observed. Peaks around 1690 cm^{-1} and 1200 cm^{-1} are vibrational modes attributed to C=O and C-O, respectively. The vibrational modes of C=N and C=C were also observed around 1500 cm^{-1} . The FT-IR spectra of the nanocomposites displayed almost all the vibrational bands inherent from PDA. As shown in **Figure 6.4a** (ii – iv) and b (ii – iv), the vibrational bands were broadened and slightly shifted to high vibrational energy for both Cu-PDA and Cu@PDA. These observations are an indication of the interaction between CuNPs and PDA functional groups. There were no observable changes on the nanocomposite's spectra as the Cu salt concentration increases. This is an indication that the incorporation of CuNPs does not affect the chemical structure of PDA.

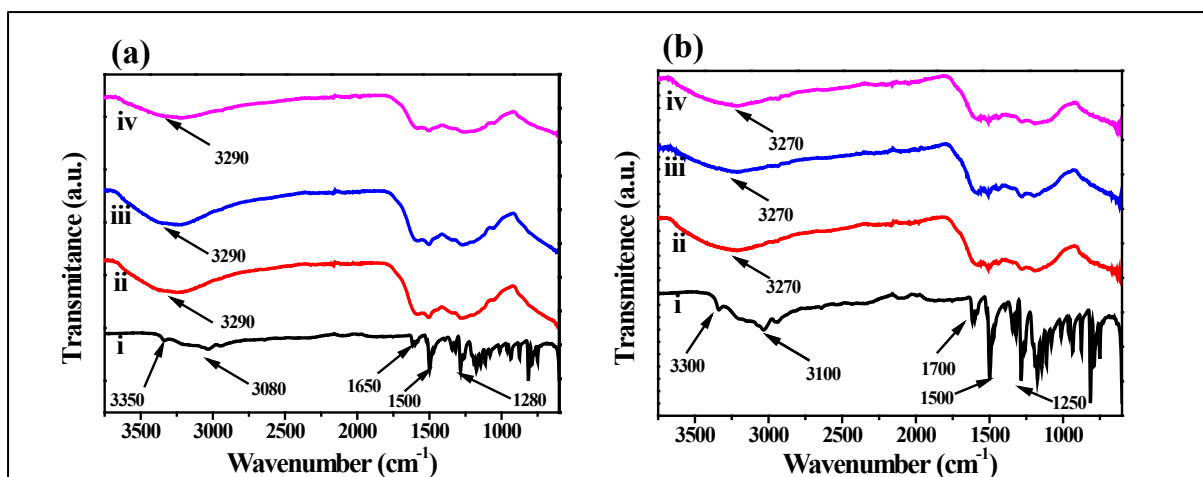


Figure 6.4: FTIR spectra of (a) Cu-PDA and (b) Cu@PDA nanocomposites at different AgNO₃ concentrations [(i) PDA, (ii) 7.4 mM, (iii) 3.7 mM and (iv) 0.74 mM].

The TGA analysis was used to conduct the thermal stability and confirm the incorporation of CuNPs on PDA. The analysis was done in air within the temperature range of 35 – 900 °C. **Figure 6.5** shows the TGA and the derivative TGA (D-TGA) thermograms of Cu-PDA (a and b) and Cu@PDA (c and d) nanocomposite. Various decomposition peaks were observed from the D-TGA of pristine PDA. This was attributed to the multi-step decomposition of PDA as a result of its heterogenous structure and the presence of various functional groups. The decomposition peak below 100 °C was attributed to the escapement of the residual moisture. The significant loss of the polymer backbone and the functional groups was noticed from the decomposition peaks around 340 °C, 500 °C and 600 °C. At 340 °C, the polymer side functional groups such as -OH and -NH were lost, and the polymer completely decomposes between 500 and 600 °C because of C=C cleavage. Upon incorporation of CuNPs, PDA lost its thermal stability. This can be seen by the shift in the decomposition peaks towards lower temperatures as shown in figure 5(a and c). The decomposition peaks below 100 °C were observed and attributed to the moisture escaping as in the pristine PDA. The functional groups of PDA in the nanocomposites were lost as early as 200 °C and 250 °C. The residual of 10%, 40% and 80% correspond to the nanocomposite prepared with 0.74, 3.7 and 74 mM of CuCl₂. The residues are due to the remaining Cu metal after PDA has completely decomposed.

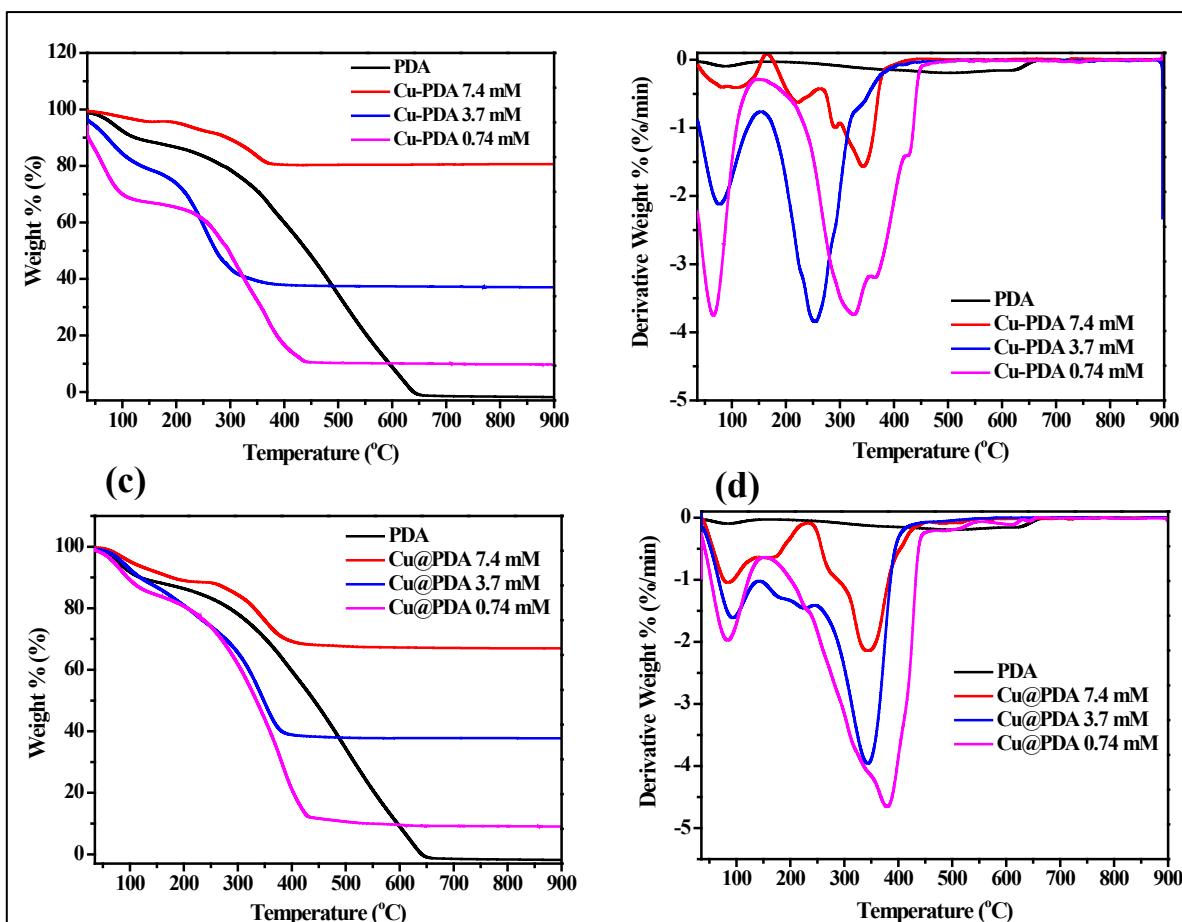


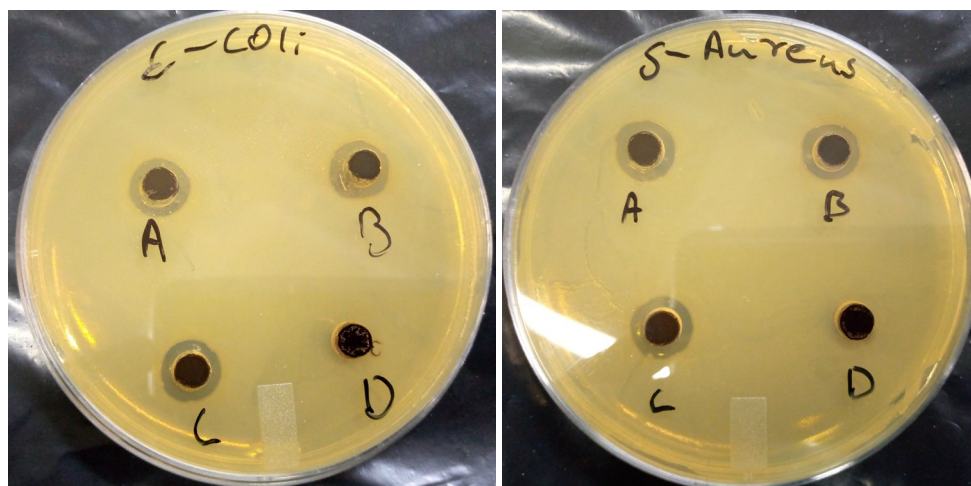
Figure 6.5: TGA and D-TGA of (a, b) Cu-PDA and (c, d) Cu@PDA nanocomposites at different CuCl₂ concentrations.

6.3.2 Antimicrobial studies

The antimicrobial activity of the nanocomposites was investigated against gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria using a standard well-diffusion method. The ability of the nanocomposites to inhibit bacterial growth was assessed by observing the clear zones of bacterial inhibition on the agar plates. **Figure 6.6** (a, b) shows images of agar plates with the bacterial inhibition zones due to Cu-PDA and Cu@PDA nanocomposites, respectively. Both nanocomposites showed promising antimicrobial properties against *E. coli* and *S. aureus* as indicated by clear zones of inhibition around the wells on the agar plates. There was no significant change in the zones of inhibitions for Cu-PDA regardless the difference in the concentrations of Cu salt and sizes of the CuNPs. A slight increase in the diameter of the zones of inhibition was observed due to Cu@PDA nanocomposite as the concentration of Cu salt and the sizes of CuNPs increase against both strains. The exposure of CuNPs on the bacteria from Cu@PDA is high compared to Cu-PDA since they are on the

surface. This may be the reason Cu@PDA possessed stronger antimicrobial activity compared to Cu-PDA. PDA alone did not possess any clear antimicrobial activity. This can clearly be seen by the absence of clear inhibition zone around the wells. In this study, CuNPs were the ones responsible for antimicrobial activity of the nanocomposite. The diameters of inhibition zones are recorded in **Table 6.1**.

(a)



(b)

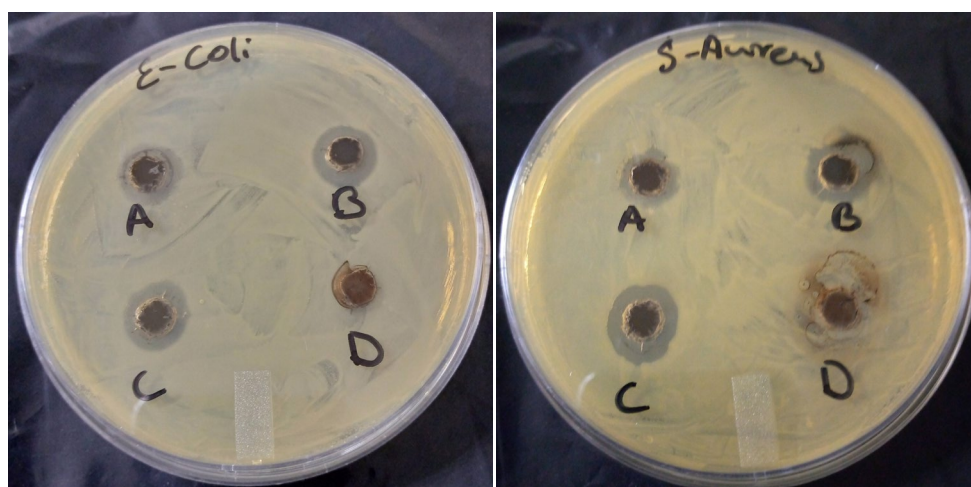


Figure 6.6: Inhibitory Zones photographs produced by (a) Cu-PDA and (b) Cu@PDA nanocomposites at different AgNO_3 concentrations [(A) 0.74 mM, (B) 3.7 mM and (C) 7.4 mM] and (D) PDA.

The minimum inhibitory concentrations (MIC) of Cu-PDA and Cu@PDA were determined to further investigate their antimicrobial activity, quantitatively. The MICs were determined through a serial dilution method and their values are recorded in Table 1. MIC values of 25 $\mu\text{g/ml}$ were obtained for all the Cu-PDA samples against both bacterial strains. MIC values

for Cu@PDA were found to be lower compared to Cu-PDA. At the lowest concentration of CuNPs loading, the MIC value for Cu@PDA was found to be 12.5 µg/mL. The MIC decreased to 6.25 µg/mL when the CuNPs loadings increase. The low MICs is an indication that less amount of Cu@PDA is required to inhibit bacterial growth compared to Cu-PDA.

Table 6.1: Summary of the zone of inhibition and MIC of the nanocomposites prepared with different concentrations of CuCl₂ (A) 0.74 mM, (B) 3.7 mM (C) 7.4 mM, and (D) PDA

Sample	Zone of Inhibition (mm)				MIC (µg/ml)			
	Cu-PDA		Cu@PDA		Cu-PDA		Cu@PDA	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
A	10	11	9	9	12.5	25	12.5	12.5
B	12	11	12	13	12.5	25	6.25	6.25
C	12	12	14	16	6.25	25	6.25	6.25
D	0	0	0	0	0	0	0	0

6.3.3 Biocompatibility studies

It is important to understand the cytotoxicity of the nanocomposites before their practical application in biomedical fields including but not limited to wound healing. Conducting cytotoxicity studies helps to understand if the nanocomposites will have negative impact in wound healing processes. Therefore, biocompatibility of the nanocomposites at different concentrations was investigated by evaluating their cytotoxicity towards BHK-21 cell line, *in vitro*. The MTS cell proliferation assay was used to determine the viability of BHK-21 cells after being treated with the nanocomposites. The effect of CuNPs loading (in terms of the CuCl₂ concentration) on the cytotoxicity was also evaluated as shown in **Figure 6.7**. There was no significant toxicity observed due to Cu-PDA (a) and Cu@PDA (b) at low concentrations. However, as the concentration of the Cu@PDA nanocomposite increased, a drastic decrease of cell viability was observed, especially due to nanocomposites prepared with 7.4 and 3.7 mM of CuCl₂. Cu@PDA prepared with 0.7 mM of CuCl₂ showed an excellent cell viability (about 80%) at nanocomposite concentration as high CuCl₂1000 µg/ml. A relatively good cell viability was observed due to Cu-PDA nanocomposites. Unlike Cu@PDA nanocomposites, a good cell viability was observed for Cu-PDA prepared with high concentration of CuCl₂ (3.7 mM). However, for Cu-PDA prepared with 7.4 mM of CuCl₂, a rapid decrease in cell viability was observed just like with Cu@PDA nanocomposites. The amount of CuNPs loading on the PDA also showed to influence the

cytotoxicity. These was indicated by a reduced biocompatibility of the nanocomposites prepared with high concentrations of CuCl₂. Nanocomposites prepared by 7.4 mM of CuCl₂ possessed a significant cytotoxicity towards the BHK-21 cells while the ones prepared with 0.7 mM were relatively non-toxic for a wide range of nanocomposites concentration (7.8 to 1000 µg/ml). However, at 3.7 loading concentration, Cu@PDA started to show a significant toxicity, especially at the concentration range of 62.5 to 1000 µg/ml. The high toxicity of Cu@PDA is attributed to the fact that CuNPs are completely exposed to the cells.

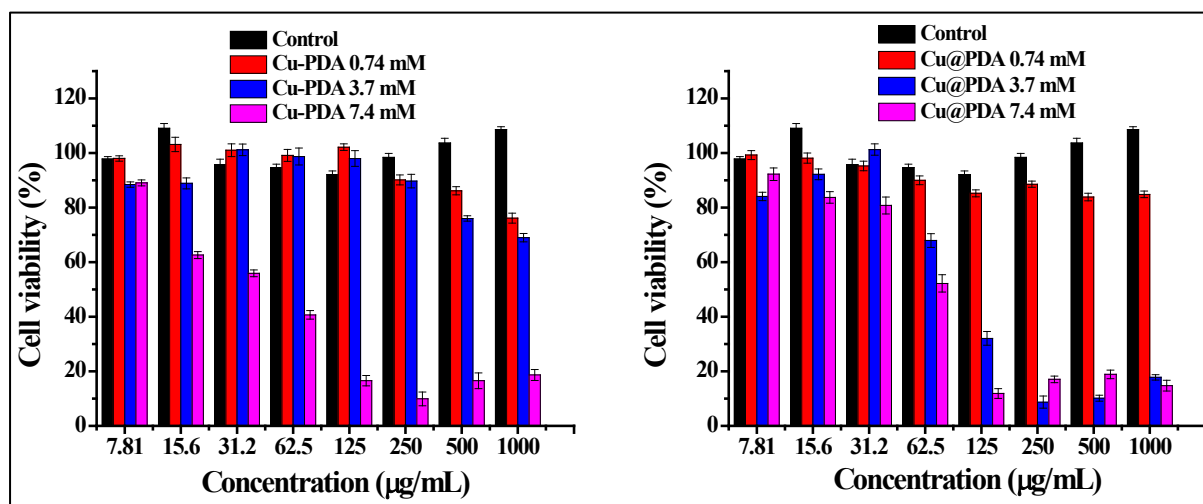


Figure 6.7: Cell viability of BHK-21 cells exposed to (A) Cu-PDA and (B) Cu@PDA at different concentrations of CuCl₂.

The minimum concentration at which the nanocomposites kill 50% of the BHK-21 cells was evaluated. This concentration is known as the 50% cytotoxic concentration (CC₅₀) and it is useful in the sense that it allows one to be able to select appropriate and non-toxic concentration of the sample prior applications. The CC₅₀ values of Cu-PDA and Cu@PDA were determined from the sigmoid curves shown in **Figure S6.2** and are recorded in **Table 6.2**. Fairly high CC₅₀ were observed which indicates that the nanocomposites are less toxic towards BHK-21 cells. Interestingly, Cu@PDA with low CuNPs loading [0.7 mM of CuCl₂] and Cu-PDA at loading concentrations of 0.74 mM and 3.7 mM did not have the CC₅₀. This is can clearly be seen in **Figure 6.7** where the cell viability was well above 50% for the entire concentration range (7.8 to 1000 µg/ml) used in this study. These observations are an indication of good biocompatibility of the nanocomposites.

The MIC and CC₅₀ values obtained for the Cu-PDA and Cu@PDA nanocomposites were used to determine their SI values (i.e., CC₅₀/MIC). This parameter is considered a safety margin and the wider it is, the safer the therapeutic agent (nanocomposites). A therapeutic

agent is considered favourable if the SI value is ≥ 10 . As shown in **Table 6.2**, the SI values obtained for the Cu-PDA nanocomposites prepared with 7.4 mM concentration was found to be 9.6 and 2.4 for both *E. coli* and *S. aureus*, respectively. On the other hand, Cu@PDA nanocomposites prepared with 3.7 and 7.4 mM AgNO₃ concentrations had SI values of 13.6 and 10.2, respectively, for *E. coli* and *S. aureus*. These results show that the Cu-PDA samples had moderate safety margins only against *E. coli* (SI close to 10), while the Cu@PDA nanocomposites showed favourable safety profiles at the tested concentrations. It is important to note that the SI values of the nanocomposites were not determined at some concentrations of CuCl₂ (i.e., 0.74 and 3.7 mM for Cu-PDA, and 0.74 mM for Cu@PDA). This is because their CC₅₀ values could not be determined due to high cell viability (over 50% for the entire concentration range) posed by the nanocomposite.

Table 6.2: Summary of the CC₅₀ and SI values obtained for Cu-PDA and Cu@PDA nanocomposites prepared with different concentrations of CuCl₂ **(A)** 0.74 mM, **(B)** 3.7 mM **(C)** 7.4 mM, and **(D)** PDA

Sample	CC ₅₀ (µg/mL)		SI value			
	Cu-PDA	Cu@PDA	Cu-PDA		Cu@PDA	
			<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
A	-	-	-	-	-	-
B	-	85	-	-	13.6	13.6
C	60	64	9.6	2.4	10.2	10.2
D	-	-	-	-	-	-

6.4 Conclusions

Cu-PDA and Cu@PDA nanocomposites were successfully designed through a facile and green synthesis method. The diameters of AgNPs were controlled by varying the concentration of CuCl₂. There was an increase in NPs diameter with increasing concentration of CuCl₂. Both the nanocomposites showed excellent thermal stability as shown from the TGA analysis. However, the thermal stability of PDA was slightly compromised after incorporating NPs. The cytotoxicity of the nanocomposites towards BHK-21 cells was relatively low. However, at high concentrations, the nanocomposites became toxic. Cu@PDA affected the cell viability than Cu-PDA, making it more toxic than the latter. Cu-PDA and Cu@PDA nanocomposites showed inhibition of bacterial growth. The MIC values obtained for both Cu-PDA and Cu@PDA nanocomposites were significantly lower than their CC₅₀ values. These results show that these materials could potentially be used as antimicrobial

agents without stimulating toxicity against mammalian cells. The antimicrobial activity of these composites against *E. coli* and *S. aureus* was outstanding. Cu@PDA nanocomposites possessed strong antimicrobial activity than Cu-PDA. These, nanocomposites pose great potential in advanced wound care treatment. This is because of their ability to kill bacteria at low concentrations and their good biocompatibility towards fibroblasts cells. Fibroblasts are the main cells present in skin connective tissue (dermis) and play a crucial role during cutaneous wound healing.

6.5 References

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Chapter 7

Facile synthesis of antimicrobial active dopamine capped silver/copper bimetallic nanofibres and their potential application in wound healing

7.1 Introduction

Metal nanoparticles (NPs) have attracted substantial research attention due to their superior physico-chemical properties. As a result, they have been applied in various fields including catalysis, electronics, environmental sciences, etc. In addition, metal NPs have also been widely used as antimicrobial agents in a wide range of products (i.e. plastic products, textiles and coating-based usages). Due to their small sizes which contribute to their high surface to volume ratio, NPs can easily penetrate the cell membrane and interact with vital organelles within microorganisms. The antimicrobial properties of silver (Ag) and copper (Cu), amongst other metal NPs, have been widely investigated. Ag and Cu NPs are well known for their broad spectrum of antimicrobial activity due to their virucidal, fungicidal and bactericidal properties. The mechanism of action of Ag and Cu NPs against bacteria and other microorganisms have been well studied and reported in literature. The ease and abundance of the synthetic methods for fabricating these NPs makes it easier to apply them. As such, Ag and Cu NPs have gained attention in biomedical applications such as drug delivery, diagnostics and wound healing. In wound healing applications, Ag and Cu NPs are responsible for inhibiting bacterial infections. They have also been reported to improve cell proliferation and promote the production of fibroblasts during the wound healing process. Like other metal NPs, Ag and Cu NPs tend to lose their stability when left in suspension, this is due to high surface energy, which ultimately leads to agglomeration. Agglomeration limits the applicability of NPs since they lose their intrinsic properties. Agglomerated NPs are also not suitable to be applied in wound healing since they have been found to be toxic towards mammalian cells. Various methods for stabilizing Ag and Cu NPs have been implemented, including the use of support materials and capping agents. Support materials such as polymers, carbon, alumina and titania have been used to improve the stability of NPs. Capping agents such as alkanethiols, cetyltrimethylammonium bromide (CTAB) and other polymers have also been used to control the stability of NPs. In addition, the use of capping agents can also control the shapes and sizes of NPs.

Synthesis of bimetallic NPs has drawn great interest in science and technology. The idea of fabricating bimetallic NPs is to combine the physical and chemical properties of the individual metals. These NPs can be synthesized in the form of alloys or core shells, and have been reported to pose unique optical, electronic and catalytic properties compared to mono-metal NPs [6]. Ag/Cu bimetallic NPs were found to possess a wide antibacterial spectrum at low concentrations due to the synergy from the respective metals. Despite previous studies on noble bimetallics and their preparation and application, a few studies have been conducted on Ag/Cu bimetallics [7-9]. The lattice constants of Ag and Cu are 0.361 and 0.409, respectively. Since they are too different, preparing bimetallics of these metals can be a challenge. It is also challenging to simultaneously reduce both Ag and Cu due to the differences in their reduction potentials (0.8V and 0.34V, respectively). Furthermore, the instability of Cu in air is an added obstacle. Incorporating Ag and Cu into Ag/Cu bimetallic NPs reduces the instability of Cu NPs in air. Additionally, bimetallics have a potential to offer a cost-effective way of noble metal NPs since only small amount is needed for preparation. In the present study, a facile and a novel approach of synthesizing Ag/Cu bimetallic NPs was reported. The bimetallic NPs in the form of nanofibres (BNF) were obtained. Possessing an excellent antimicrobial activity, the BNF were further tested for their potential in wound healing. The cytotoxicity of the BNF was tested using baby hamster kidney (BHK-21) 1fibroblasts *in vitro*. The bimetallics were found to be biocompatible with low cytotoxicity towards skin cells. In addition to the excellent antimicrobial activity, these BNFs showed a good potential in wound healing activity due to their non-toxic nature against skin cells.

7.2 Experimental

7.2.1 Materials

Silver nitrate (AgNO_3 , 99.0%), Copper Chloride (CuCl_2 , 99.9%), Dopamine hydrochloride, Tris buffer, Luria Bertani Broth (LB), Tryptic Soy Agar (TSA), dimethylformamide (DMF, 99.99%), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS) and penicillin-streptomycin were all purchased from Sigma Aldrich and used as received. All glassware was washed with an aqua-regia solution and rinsed with distilled water several times.

7.2.2 Synthesis of bimetallic nanofibres

To synthesize Cu/Ag bimetallic nanofibres, the mixture of various amounts of CuCl_2 and AgNO_3 were dissolved in 100 mL distilled water. This was followed by addition of dopamine hydrochloride (50 mg) to the mixture. The solution was then heated to boil (98 °C). To the boiling solution, 5 mL of hydrazine hydrate (3%) was added. The reaction was further carried out for 1 h. To vary the amount of each metal in the nanofibres, Cu:Ag mole ratios of 1:0, 0:1, 1:1, 1:2 and 2:1 were used. It is important to note that when 1:0 or 0:1 mole ratio was used, either CuCl_2 or AgNO_3 was excluded. Prior characterization, the product was cooled, washed using distilled water with the aid of centrifugation.

7.2.3 Cell viability studies

Please refer to section 3.2.3 for detailed procedure. The final concentrations of the materials were ranging between 7.8-1000 $\mu\text{g/mL}$.

7.2.4 Antimicrobial activity tests

7.2.4.1 Well diffusion method

Please refer to section 3.2.4.1 for detailed procedure.

7.2.4.2 Minimum inhibitory concentration (MIC) and bacteriostatic studies

Please refer to section 3.2.4.2 for detailed procedure. The concentration range of the bimetallic was 100 to 0.78125 $\mu\text{g/mL}$.

7.2.5 Characterization

All excess impurities from colloidal solutions were removed using a universal 320R centrifuge at a maximum speed of 15000 rpm for 15 minutes. Chemical structure analysis was done using a Bruker Tensor 27 Fourier transform infrared (FTIR) spectrometer. Thermal analysis was conducted using a Perkin Elmer TGA 6000 thermogravimetric analyser (TGA) using high-purity nitrogen at a heating rate of 10 °C/min and gas flow rate of 10 mL/min. Transmission electron microscopy (TEM) images were obtained from FEI Tecnai-T12

microscopy at an electron acceleration voltage of 120 kV and beam spot size of 10-100 nm. The TEM particle diameters were determined using ImageJ software. Powdered X-ray diffraction (PXRD) analysis was performed on a Bruker D2 Phaser with Cu radiation and each scan was recorded over 20 min.

7.3 Results and discussion

7.3.1 Materials characterization

The chemical synthesis of Cu/Ag bimetallic nanofibres (Cu/Ag BNF) was accomplished by core reduction of Cu and Ag ions with hydrazine, in the presence of dopamine hydrochloride as a capping agent. The optical properties of the bimetallic was elucidated by UV-vis absorption spectroscopy. **Figure 7.1** shows the absorption spectra of Cu/Ag BNF prepared with different mole ratios of Cu and Ag ions. When mole ratio of 1:0, this is when only 1 mmol of Cu precursor was used, a broad absorption band was observed with maximum absorption at the wavelengths of 590 nm. This absorption band is attributed to the surface plasmon resonance (SPR) phenomenon due to the coherence oscillation of conduction electrons on the surface of copper nanoparticles (CuNPs). In addition, the presence of this absorption band is also a confirmation of the successful synthesis of CuNPs. In the case where the mole ratio of 0:1 was used, meaning only 1 mmol of Ag precursor was present during the synthesis, a broad absorption band with the absorption maximum at the wavelength of 415 nm was observed. This absorption band represent the SPR and also confirms a successful synthesis of AgNPs. A much broader absorption band was observed when both Cu and Ag with the mole ratio of 1:1 (Cu:Ag) were added in the reaction mixture. This broad band is attributed to the SPR properties of both Cu and Ag which confirm the presence of Cu/Ag BNF. As the mole ratio was changed to 1:2, the absorption band split into two distinct bands, which clearly indicates the presence of both Cu and Ag. When 2:1 mole ratio was used, two absorption bands were also observed, with the Cu SPR band more dominant than that of Ag. This is due to high amount of Cu ions used compared Ag ions. Same observations were made when more Ag ions (1:2) were used. It is important to note that all the SPR bands were broad which could be due to agglomeration of the BNF or wide size distribution.

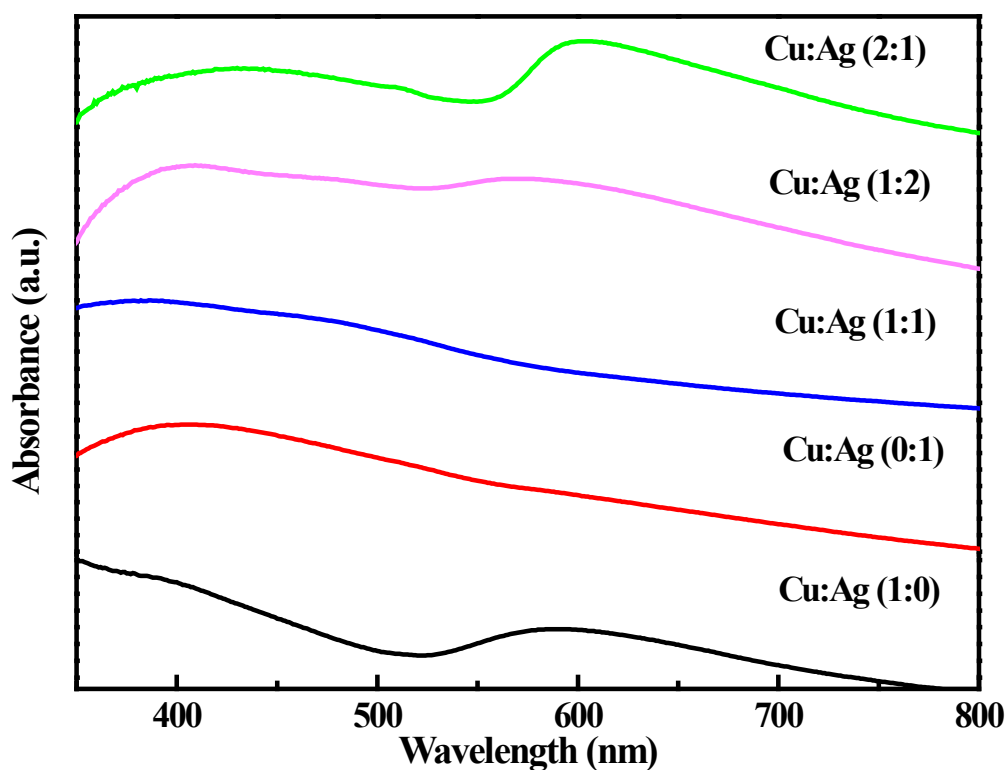


Figure 7.1: UV-vis spectra of BNF at various mole ratio of Cu to Ag.

The morphological analysis of Cu/Ag BNF was conducted using TEM. **Figure 7.2** shows the TEM micrographs of the synthesized BNF at various mole ratios of precursors. **Figure 7.2** (a, b) shows the TEM micrographs of the independently prepared Cu (1:0) and Ag (1:0) NPs, respectively. Spherical-like Cu and Ag NPs were obtained with average diameters of 22 and 11 nm, respectively. The size distribution histograms of both Cu and Ag NPs are shown in **Figure S1**. It is important to note that Cu and Ag NPs appeared to have been supported on a network or a cluster of polydopamine. The zoomed TEM micrograph that clearly shows the polydopamine network is shown in **Figure 7.2c**. The polydopamine network or clusters are associated with the partial polymerization of dopamine. The partial polymerization of dopamine can be attributed to the presence of hydrazine hydrate that was used to reduce Cu and Ag ions. Hydrazine hydrate is a also strong base, which offers the best condition for self and partial polymerization of dopamine.

A change in morphology was observed after mixing both Cu and Ag precursors for bimetallic fabrication. Fibrous morphologies of Cu/Ag BNF prepared with Cu:Ag mole ratios of 1:1 (BNF-1), 1:2 (BNF-2) and 2:1 (BNF-3) are shown in **Figure 7.2d, e and f**, respectively. The BNF appeared to have been coated with a small but significant trace of polydopamine on the sides. In addition, polydopamine networks that get formed during synthesis process, are the

ones responsible for controlling the growth on the NPs into the formation of the BNF. At 1:1 mole ratio (Figure 2d), pure BNF were formed with some lumps (circled) where the fibres interconnect. As the mole ratio changes to 1:2, the fibres appeared to be more elongated with few lumps and NPs agglomerates on the sides. The fibres got even more elongated when at the mole ratio of 2:1, however they were mixed with spherical NPs on the sides. The presence of agglomerates and spherical NPs at 2:1 and 1:2 mole ratios could be attributed to high concentration of either Cu or Ag ions. At high concentrations, some of the ions will not partake in the formation of fibres during nucleation and growth process because of oversaturation, hence the formation of separate NPs or agglomerates.

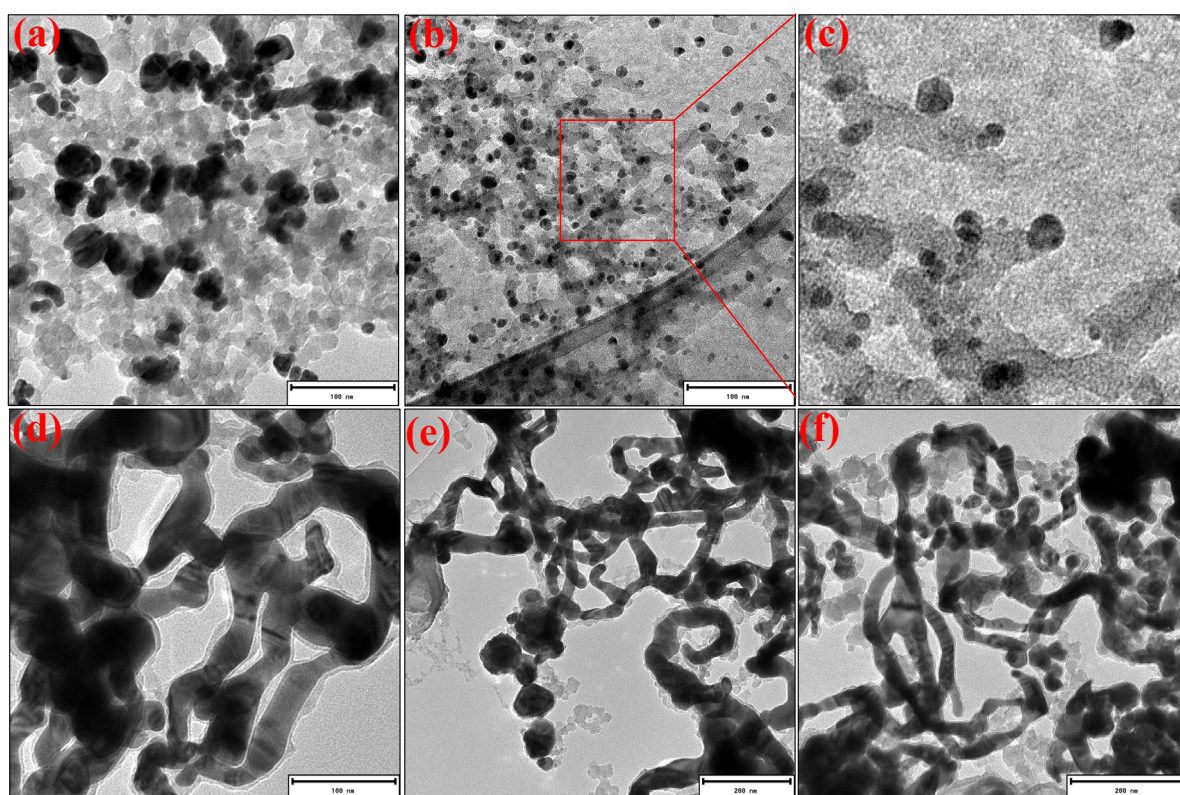


Figure 7.2: TEM micrographs of BNF at various mole ratios of Cu to Ag. (a) 1:0, (b) 0:1, (c) zoomed micrograph of (b), (d) 1:1, (e) 1:2 and (f) 2:1.

The BNF crystal phase structure and the crystallinity analysis were conducted using PXRD. **Figure 7.3** represent the diffractograms of Ag NPs, Cu NPs and BNF-1 to BNF-3. The diffractograms of Cu NPs (black) shows the three diffraction peaks at the diffraction angles of 44° , 55° and 74° which correspond to (111), (200) and (220) lattice plains of face centred cubic (FCC) phase of metallic Cu. Five diffraction peaks at diffraction angles of 38° , 45° , 65° , 77° and 83° were observed from the diffractogram of Ag NPs (Red). These peaks

represent the (111), (200), (220), (311) and (222) lattice planes of FCC phase of metallic Ag. The absence of foreign peaks in Cu and Ag NPs diffractograms signifies the formation of pure metallic Cu and Ag NPs.

The diffraction peaks that correspond to both metallic Cu and Ag were observed from the diffractograms of BNF-1 (Blue), BNF-2 (Magenta) and BNF-3 (Lime). The presence of all the diffraction peaks representing both the lattice plains of Cu and Ag in one diffractogram is an indication of the formation of Cu/Ag BNF. A complete overlap of Cu (111) and Ag (200) crystal plains was observed from BNF-1 diffractogram. As the mole ratio of Cu:Ag were altered (BNF-2 and BNF-3), a slight split of Cu (111) and Ag (200) peak was observed, with an obvious split more observable on BNF-3 diffractogram. The splitting of this peak can be attributed to the formation of the mixture of both the Cu/Ag alloy and either Cu or Ag NPs. This was supported by the PDF no's. which respectively matched to both Cu, Ag and Cu/Ag alloy (BNF-1 to BNF-3). Surface area selected diffraction (SAED) was further utilized to study the nature of crystallinity of the materials. **Figure S7.1** shows the SAED images of (a) pristine Cu, (b) pristine Ag and (c) BNF-1. The pristine Cu showed discrete spots forming a distinct ring pattern which indicate a polycrystalline nature of the materials. The single crystal nature of the pristine Ag was observed as the diffraction pattern was only characterised by discrete spots indicating that all the atoms are in the same order for this sample. Due to the random arrangement of the crystal lattice of both Ag and Cu, the BNF-1 was found to be polycrystalline in nature.

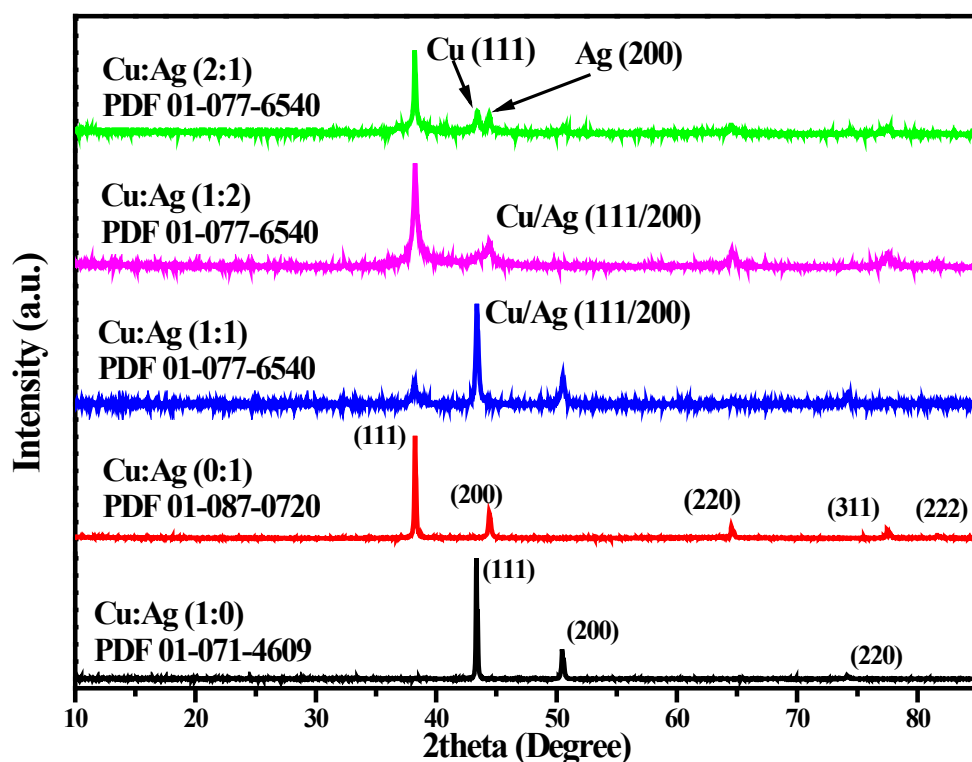


Figure 7.3: PXRD patterns of BNF at various mole ratio of Cu:Ag.

7.3.2 Antimicrobial studies

The antimicrobial activity of the pristine (Cu and Ag NPs) and the BNF was investigated against *E. coli* and *S. aureus* using a standard well-diffusion method. Images of the agar plates showing clear zones around the wells are presented in **Figure 7.4**. The clear zones around the wells indicate the sensitivity of the bacteria to the NPs and the BNFs. Ag NPs exhibited a strong bacterial growth inhibition compared to Cu NPs and the BNF against both *E. coli* and *S. aureus* strains. This is because the wells containing Ag NPs are surrounded by large zones of inhibition compared to those with Cu NPs and BNF. The strong antimicrobial activity of Ag NPs compared to other materials can be attributed their small sizes. The average diameter of Ag NPs was almost half that of Cu NPs, and the BNFs are also large in terms of aspect ratios. BNF-1 showed the smallest inhibition zone compared to BNF-2 and BNF-3, with BNF-3 showing the highest against both *E. coli* and *S. aureus*. The aspect ratios of BNF-2 and BNF-3 appeared to be large compared to BNF-1. In addition, mixtures of either Ag or Cu NPs and the alloy were obtained in BNF-2 and BNF-3 which may have played a role in their antimicrobial activity. The existence of errors during the measurement of zones of inhibition diameter are acknowledged since a ruler with the resolution of 1 mm

was used. The measured zones of inhibition are recorded in **Table 7.1**. The minimum inhibitory concentration (MIC) of the materials was determined and recorded in **Table 7.1**. Ag NPs and BNF-3 displayed lower MICs than Cu NPs, BNF-1 and BNF-2. This is a clear indication that both *E. coli* and *S. aureus* are more sensitive to Ag NPs and BNF-3. The observations made from the MICs were in support to the findings from the well diffusion analysis.

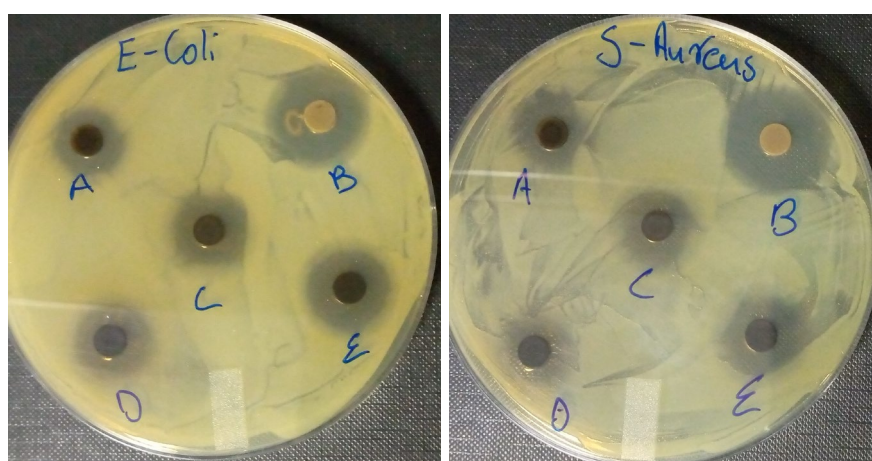


Figure 7.4: Visible inhibitory Zones produced by (A) Cu NPs, (B) Ag NPs, (C) BNF-1, (D) BNF-2 and (E) BNF-3 against (a) *E. coli* and (b) *S. aureus*.

Table 7.1: Summary of the zone of inhibition and MIC of (A) Cu NPs mM, (B) Ag NPs (C) BNF-1, (D) BNF-2 and (E) BNF-3

Sample	Zone of Inhibition (mm)		MIC ($\mu\text{g/ml}$)	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
A	13	13	15	15
B	22	23	7.5	7.5
C	14	13	15	15
D	16	16	15	15
E	20	19	7.5	7.5

7.3.3 Biocompatibility studies

Any material that is to be applied in wound treatment should be biocompatible or at least not consist or release harmful toxins that can hinder the wound healing process. The

biocompatibility study of the NPs and BNFs was carried out by *in vitro* evaluation of their cytotoxicity towards BHK-21 cells. Fibroblasts are cells that play a vital role in cutaneous wound repair by producing collagen, which is the main structural component of the Extracellular matrix (ECM). The cell viability of BHK-21 cells after treatment with the samples was conducted using the well-known MTS cell proliferation assay. After being treated with the samples, the cells were further treated with MTS dye. The viable cells metabolize the MTS into a purple soluble formazan. The number of live cells and their concentrations was indicated by contrast of the purple formazan which were quantitatively determined using UV spectroscopic plate reader. The percentage (%) of viable cells was determined in reference to the control or untreated cells. As Evident from **Figure 7.5**, BNF-1, BNF-2 and BNF-3 showed insignificant cytotoxicity towards BHK-21 cells at low concentration (7.8 µg/mL) with cell viabilities ranging between 75-90%. BNF-1 maintained its good cell viability of over 80% at concentration as high as 62.5 µg/mL. The cell viability drastically decreased below 50% due to BNF-3 with the concentration of 62.5 µg/mL. A drastic decrease of cell viability due to BNF-3 can be associated with the high concentration of Cu NPs. Pristine Cu NPs showed low cell viability compared to other samples. The cell viability due to Cu NPs was 60% at the lowest concentration (7.8 µg/mL). Ag NPs on the other hand showed a relatively good biocompatibility as the cell viability of over 50% was observed at relatively high concentration of 31.25 µg/mL. It is worth noting from this data that combination of Ag and Cu can be very crucial in terms of reducing their toxicity. It was clear that the cytotoxicity of BNF-1 (where 1:1 mole ratio of Cu to Ag was used) was reasonably low at concentration as high as 250 µg/mL.

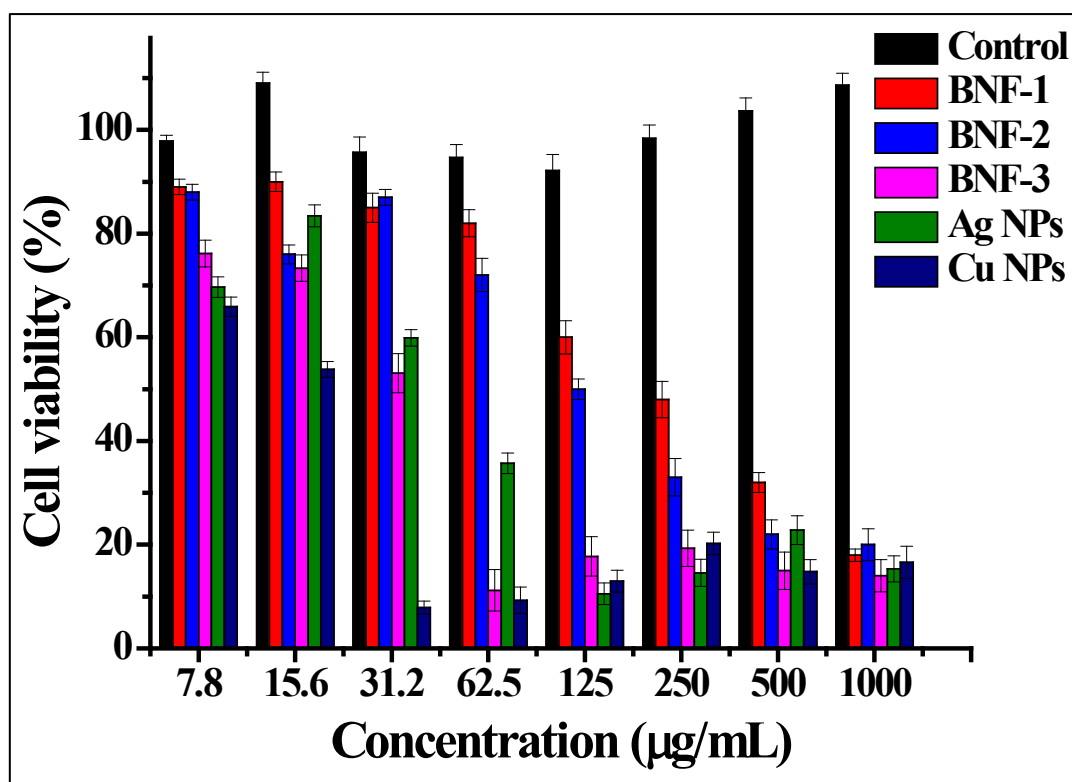


Figure 7.5: Cell viability of BHK-21 cells exposed to Cu NP, Ag NPs and the BNFs at different concentrations.

Table 7.2: Summary of the CC₅₀ and SI values obtained for the synthesized materials

Sample	CC ₅₀	SI value	
		<i>E. coli</i>	<i>S. aureus</i>
Cu NPs	18.5	1.2	1.2
Ag NPs	51.3	6.8	6.8
BNF-1	201.1	13.4	13.4
BNF-2	117.8	7.9	7.9
BNF-3	50.7	6.8	6.8

The 50% cytotoxic concentrations (CC₅₀) of the as-synthesized materials were determined in order to evaluate the applicable concentrations during wound healing. The CC₅₀ of Cu NPs, Ag NPs, BNF-1, BNF-2 and BNF-3 were determined from the sigmoid curves shown in **Figure 7.6** and are recorded in **Table 7.2**. Relatively high CC₅₀ values were obtained for BNF-1 and BNF-2, indicating that they are less toxic towards BHK-21 cells. These

observations are an indication of adequately good biocompatibility of BNF-1 and BNF-2. The CC₅₀ of BNF-3, Ag NPs and Cu NPs were relatively low with Cu NPs having the lowest. Using the MIC and CC₅₀ values obtained for the BNF samples, the selectivity index (SI) values of these materials were calculated. As shown in Table 7.2, the SI values obtained for the Cu NPs, Ag NPs, BNF-1, BNF-2 and BNF-3 were 1.2, 6.8, 13.4, 7.9 and 6.8, respectively, against both *E. coli* and *S. aureus*. These results show the Cu NPs to be the least safe, followed by the Ag NPs, BNF-3 and BNF-2; with BNF-1 being the only ones that presented favourable safety margins.

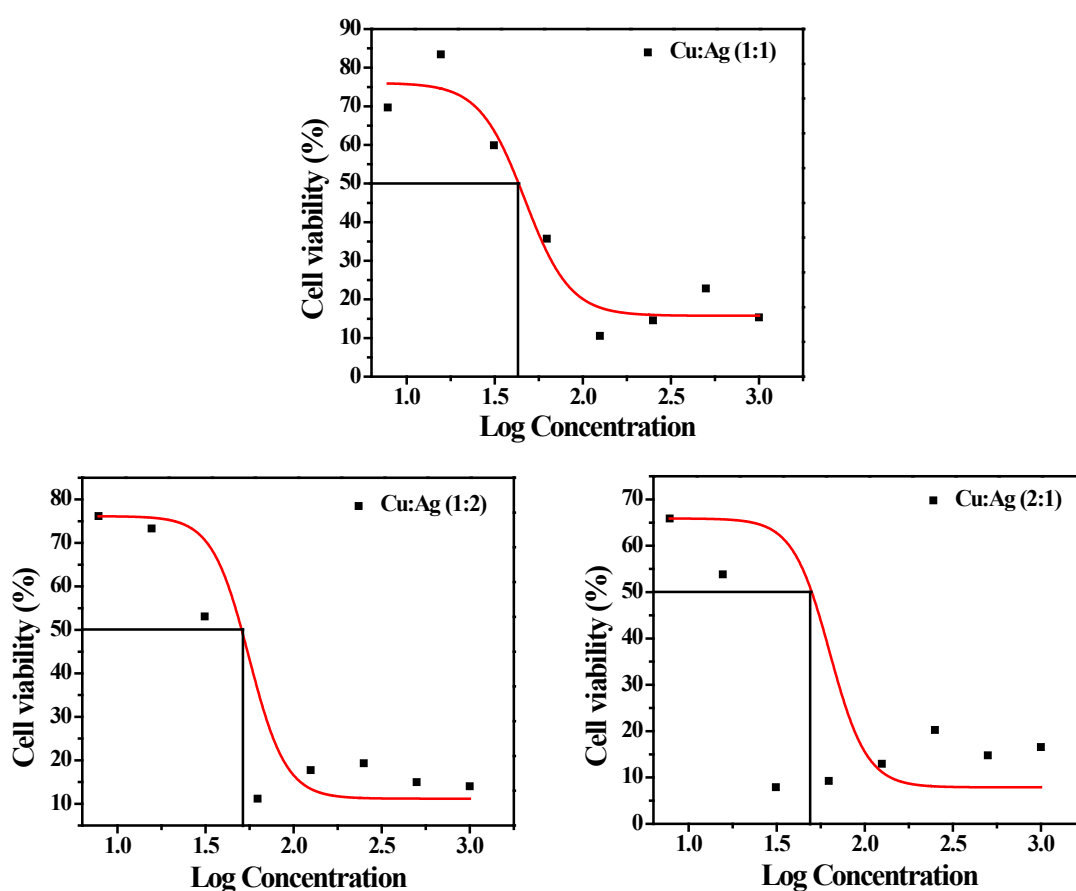


Figure 7.6: The sigmoid curves of cell viability acquired by incubating BHK-21 cells with BNF-1, BNF-2 and BNF-3 at different concentrations.

7.4 Conclusions

The BNFs were successfully synthesized using hydrazine and dopamine a reducing and capping agent, respectively. Dopamine, which partially polymerizes due to the presence of hydrazine played a big role in controlling the shape of the resultant BNFs. There was no clear effect on the morphology of the BNFs due to varying Cu:Ag mole ratios. However, at mole

ratio of 1:2 and 2:1, the mixture of nanofiber and spherical nanoparticles were obtained, evident from TEM analysis. Pure nanofibres were obtained when 1:1 mole ratio was used. PXRD analysis confirmed the alloy nature of the BNFs, and no other impurities (i.e., oxides) were observed from the diffractograms. The BNFs exhibited substantial antimicrobial activity against *E. coli* and *S. aureus*, with BNF-3 proving to be a stronger antimicrobial agent, followed by BNF-2, then BNF-1. This was indicated by big inhibition zone and low MIC possessed by BNF-3. In the biocompatibility studies, however, BNF-3 (together with BNF-2) showed unfavourable SI values that were less than 10. With BNF-1 being the only sample that had a favourable SI value. Given BNF-1's poor activity against both *E. coli* and *S. aureus*, further modifications are required to improve its potency.

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Chapter 8

Polydopamine coated gelatin/chitosan scaffolds decorated with silver and copper nanoparticles for application in wound healing

8.1 Introduction

Recent developments in advanced technology and nanotechnology have proved promising solutions in therapeutics, including wound care management [1, 2, 3]. Advanced wound dressings in hydrocolloids, films, foams, hydrogels and scaffolds have been designed and used to solve health problems underlying wound care treatments [4, 5, 6, 7, 8, 9, 10, 11, 12, 13]. These wound dressings are designed to meet ideal requirements which include the ability to maintain a balanced moist environment, adherence to the wound, allow the exchange of oxygen and prevent bacterial infection. The requirements ensure the acceleration of wound healing and reduce the complications that are associated with the process. Wound dressings are designed as either temporary or permanent dressings [14, 15, 16, 17, 18, 19, 20]. Temporary wound dressings provide, and control wound exudate absorption and protect them until closure. They also prevent or protect wounds from bacterial infection provided they contain an antimicrobial agent. These dressings are most suitable for superficial wounds, and they require to be changed to avoid bacterial contamination [14]. Permanent dressings on the other hand tend to integrate with the skin and accelerate the tissue regeneration process. These dressings are designed in the form of tissue scaffolds that influence cell migration, adhesion and proliferation to promote epidermal regeneration and reduce scarring [20, 18, 19].

Tissue scaffolds are a 3D synthetic framework or electrospun nanofibrous mats made of biomaterials that are suitable for deep chronic wounds that are too complicated to heal through conventional treatments. They are usually designed from natural and/or synthetic polymers. Only biocompatible and biodegradable polymers are used for the fabrication of tissue scaffolds. This is because the scaffolds are embedded in the wound and integrate with the skin tissue, therefore their toxicity must be thoroughly controlled. Synthetic polymers that have proven to be the best candidates for skin tissue scaffolds include poly(glycolic acid) (PGA) [10], polyethylene glycol (PEG) [11], poly(caprolactone) (PCL) [12], poly(lactic-co-

glycolic) acid (PLGA) [13], polyvinylpyrrolidone (PVP) and polyurethane (PU) [21, 22, 23, 24, 25, 26, 27]. These polymers are biocompatible, nontoxic, structurally stable and biodegradable which make them suitable to be applied in skin tissue fabrication. Natural polymers are derived from natural sources like plants and animals. By nature, they are biocompatible and biodegradable which makes them even more suitable for designing skin tissue scaffolds. The most used natural polymers in skin tissue scaffolds include alginate, dextran, chitosan, collagen, and gelatine [28, 29, 30, 31, 32, 33].

One of the main requirements for skin tissue scaffolds is the ability to prevent bacterial infection. However, some natural and synthetic polymers are not antimicrobial active which is one of the drawbacks when it comes to wound healing. Bacterial infection is still a formidable issue in the wound healing process. Various approaches to dealing with a bacterial infection in the wound healing process have been reported [34, 35, 36]. These include incorporating antibiotics and plant extracts, or antimicrobial active metal nanoparticles (NPs) on the scaffolds [37, 38, 39, 40]. Metal NPs such as silver (Ag) and copper (Cu) have been widely studied as antimicrobial agents in wound dressing. Ag and Cu NPs are known for their broad spectrum of antimicrobial activity against bacteria, viruses and fungi. Incorporating antimicrobial active components such as Ag and Cu NPs into tissues scaffolds makes them a more suitable solution for complicated chronic wound healing [41, 42, 43, 44, 45]. This project focussed on the development of active tissue scaffolds that can be applied in the treatment of deep chronic wounds. The scaffolds were designed using nanocomposites that contain biocompatible and biodegradable polymers, together with proteins decorated with antimicrobial active nanomaterials such as Cu and Ag NPs. Cu and Ag NPs are known to possess a fast and broad spectrum of antimicrobial activity against gram-(positive and negative) bacteria [46, 47]. Additionally, these NPs are also non-toxic to mammalian cells at regulated concentrations rendering them suitable for wound healing applications. Incorporating Cu and Ag NPs on the surface of biocompatible polymers such as polydopamine (PDA), gelatine and chitosan will further improve their applicability in wound healing. PDA has been widely used in biomedical applications, including wound healing and as an antioxidant, due to its biocompatibility and non-toxic properties [48, 49, 50, 51]. It can also adhere to various kinds of surfaces due to the presence of catechol and amine functional groups [52, 53]. Taking advantage of these properties, we showcased here for the first time the coating of lyophilized chitosan/gelatine scaffolds with PDA. We further showed a simple and effective route of incorporating Cu and Ag NPs within the matrix of the scaffolds *in-situ*.

The presence of PDA coating layer containing functional groups with the ability to reduce metal ions and facilitate good polymer/metal interaction and NPs dispersion of the polymer surface was utilized as an advantage. Chitosan has been reported to be an effective component in wound healing more especially at the beginning due to its haemostatic effects. Moreover, chitosan is antimicrobial active, and this will offer synergistic effects when incorporated with Cu and Ag nanoparticles. Gelatine on the other hand is composed of peptides sequence which is very important for cell adhesion. In addition, gelatine can easily absorb exudates and control a moist environment thus promoting the wound healing process. Therefore, these scaffolds will not only prevent bacterial infection but will also promote the development of new skin tissue, thus promoting a fast-healing process.

8.2 Experimental

8.2.1 Materials

Chitosan (CS), gelatine (Gel), genipin, silver nitrate (AgNO_3), copper chloride (CuCl_2), dopamine hydrochloride, glacial acetic acid (99.0%), ammonium hydroxide (NH_4OH , 24-26%), **Luria Bertani Broth (LB)**, Tryptic Soy Agar (TSA), dimethylformamide (DMF, 99.99%), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS) and penicillin-streptomycin were all purchased from Sigma Aldrich and used as received. All glassware was washed with an aqua-regia solution and rinsed with distilled water several times.

8.2.2 Synthesis of scaffolds

The synthesis of polydopamine (PDA) coated chitosan/gelatine scaffolds decorated with a silver (Ag), or Copper (Cu) nanoparticles (NPs) was conducted following the reaction scheme shown in **Figure 8.1**. In brief, 1.5 g of CS was dissolved into 2% (v/v) glacial acetic acid. To the CS solution, 1% (w/v) of genipin was added while stirring for 2 h. This was then followed by the addition of 1 g of Gel which was pre-dissolved in water. The mixture was stirred for 2 more hours at room temperature to crosslink the two polymers. The colour of the blend changed from clear to light pink, confirming the crosslinking of the two polymers. To coat CS/Gel with PDA, 0.1 g of dopamine dissolved in distilled water was added to the crosslinked CS/Gel mixture. This was followed by the addition of 0.1 mL of NH_4OH solution to create an alkaline condition for dopamine to self-polymerize. The reaction was carried out for 24 h. The colour of the mixture changed from white to black indicating the formation of PDA. CS/Gel and PDA/CS/Gel solutions were immediately refrigerated at $-80\text{ }^\circ\text{C}$ for 24 h.

The frozen polymers were then lyophilized for 24 h to form CS/Gel and PDA/CS/Gel 3 dimensional (3D) scaffolds.

To incorporate Cu and Ag NPs within the PDA/CS/Gel structure, *in situ* reductions of AgNO_3 and CuCl_2 was conducted. Either a solution of AgNO_3 or CuCl_2 (0.5 and 1 mmol) was added in PDA/CS/Gel solution. The mixture was stirred for 30 min while stirring at room temperature. Only Ag NPs were obtained without the addition of an access reducing agent. Catechol and amine groups from PDA were only able to completely reduce Ag ions to form Ag@PDA/CS/Gel. To obtain Cu NPs, access hydrazine hydrate (1%) was added to the mixture while stirring for another 30 min. Upon reactions completion, the final solutions were then refrigerated at $-80\text{ }^\circ\text{C}$ for 24 h, followed by lyophilization for another 24 h to obtain Ag1/PDA/CS/Gel (0.5 mmol AgNO_3), Ag2/PDA/CS/Gel (1 mmol AgNO_3), Cu1/PDA/CS/Gel (0.5 mmol CuCl_2) and Cu2/PDA/CS/Gel (1 mmol CuCl_2) 3D scaffolds.

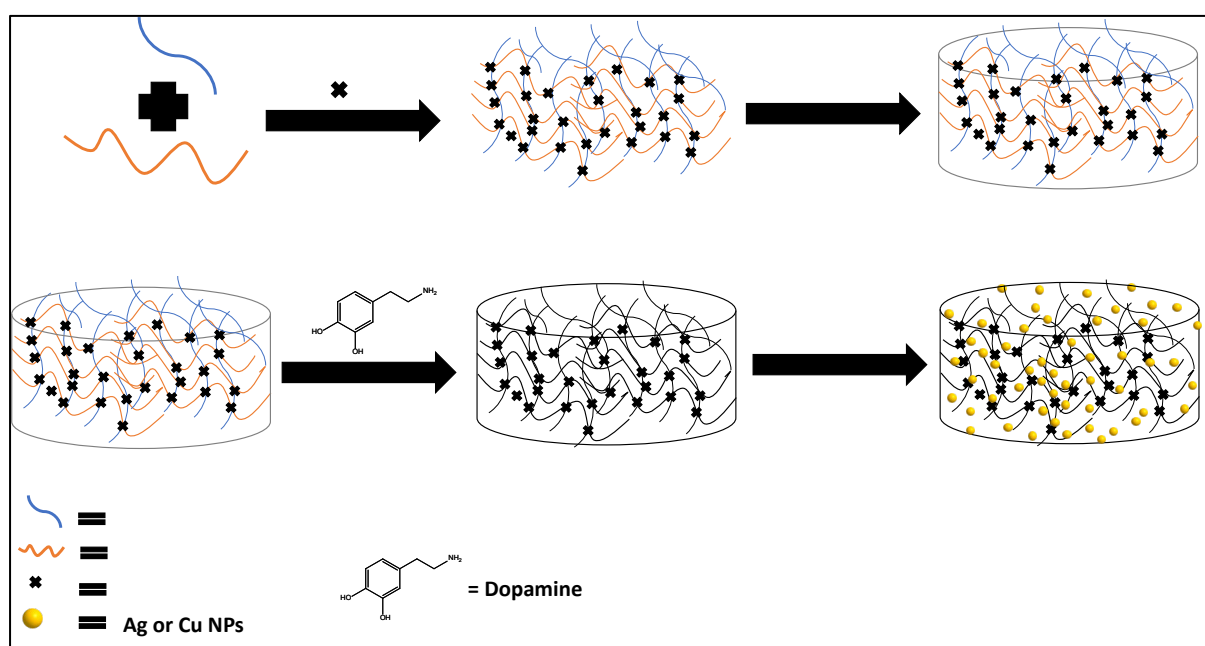


Figure 8.1: Schematic diagram showing the synthetic route of 3D Ag and Cu NPs incorporated scaffolds.

8.2.3 Swelling studies

To determine the fluid uptake (FU) of the scaffolds, swelling studies were performed in PBS (7.4%) and ultra-pure water at $37\text{ }^\circ\text{C}$. A 100 mg (W_d) of each scaffold was added in separate glass vials followed by the addition of 2 mL of PBS solution. The vials were then incubated at $37\text{ }^\circ\text{C}$. The FU was recorded every 20 min for 2 h, and then after 24 h and 48 h. Before

recording the FU, the surface absorbed PBS was removed by a filter paper. The wet weight (W_w) of the scaffolds was recorded and the % FU was determined using the equation below:

$$\%FU = \left(\frac{W_w - W_d}{W_d} \right) \times 100$$

where %FU stands for percentage fluid uptake, W_w for wet weight and W_d for dry weight as stated above.

8.2.4 Degradation studies

The degree of biodegradability of the scaffolds was determined over 14 days. A 100 mg (W_o) of each scaffold was added in a glass vial followed by 2 mL of PBS. One vial was removed each day and the remaining scaffolds were dried over 72 h and their weights were recorded (W_f). The degradability of the scaffolds was then evaluated using the equation below:

$$\%Degradation = \left(\frac{W_o - W_f}{W_o} \right) \times 100$$

8.2.5 Cell viability studies

Please refer to section 3.2.3 for detailed procedure. The final concentrations of the scaffold were ranging between 7.8 – 1000 $\mu\text{g/mL}$.

8.2.6 Antimicrobial activity tests

8.2.6.1 Well diffusion method

Please refer to section 3.2.4.1 for detailed procedure.

8.2.6.2 Minimum inhibitory concentration (MIC) and bacteriostatic studies

Please refer to section 3.2.4.2 for detailed procedure. The concentration range of the scaffolds was 300 to 2 mg/mL.

8.2.7 Characterization

All excess impurities from colloidal solutions were removed using a universal 320R centrifuge at a maximum speed of 15000 rpm for 15 minutes. Chemical structure analysis was done using a Bruker Tensor 27 Fourier transform infrared (FTIR) spectrometer. Thermal analysis was conducted using a Perkin Elmer TGA 6000 thermogravimetric analyser (TGA) using high-purity nitrogen at a heating rate of 10 $^{\circ}\text{C/min}$ and gas flow rate of 10 mL/min. Transmission electron microscopy (TEM) images were obtained from FEI Tecnai-T12 microscopy at an electron acceleration voltage of 120 kV and beam spot size of 10-100 nm. The TEM particle diameters were determined using ImageJ software. Powdered X-ray diffraction (PXRD) analysis was performed on a Bruker D2 Phaser with Cu radiation and each scan was recorded over 20 minutes.

8.3 Results and discussion

8.3.1 Characterization of the scaffolds

The bioactive scaffolds were obtained by coating the cross-linked CS/Gel with PDA followed by *in situ* decoration with either Ag or Cu NPs. The optical images of the scaffolds are shown in **Figure 8.2**. The pristine CS and Gel scaffolds were characterized by white colour, while the crosslinked CS/Gel appeared to be light pink. The pink colour was a confirmation of successful crosslinking of CS and Gel. The coating of CS/Gel resulted in the change in colour to black which was an indication of the successful formation of PDA coating the CS/Gel scaffolds. The incorporation of the scaffolds did not have any influence on the colour of the scaffolds.



Figure 8.2: Photographed images of (1) CS, (2) Gel, (3) CS/Gel, (4) PDA/CS/Gel, (5) AgI@PDA/CS/Gel and (6) CuI@PDA/CS/Gel.

The chemical structures of the pristine (CS and Gel), cross-linked CS/Gel, PDA coated (PDA/CS/Gel) and the NPs' incorporated scaffolds were studied using FT-IR spectroscopy.

Figure 8.3 shows the FT-IR spectra of the scaffolds with their characteristic functional groups. The FT-IR spectrum of CS displayed a broad and a small vibrational band around 3250 cm^{-1} and 2900 cm^{-1} , attributed to the overlap of amines (NH) and the hydroxyl (OH) stretching modes, and the aliphatic C-H stretch, respectively. The spectrum also displayed peaks around 1634 and 1555 cm^{-1} which correspond to the amide I and amide II vibrational modes ($-\text{CH}_3-\text{C}=\text{O}$). The band around 1446 cm^{-1} was attributed to the symmetrical deformation mode of CH_3 , while the one at 1246 cm^{-1} was attributed to OH groups. The vibrational modes associated with the saccharide units of CS are represented by bands around

891 and 1147 cm^{-1} . The bands around 1073 cm^{-1} and 1023 cm^{-1} were attributed to C-N and C-O stretching modes.

The FT-IR spectrum of Gel also displayed vibrational bands around 3250 cm^{-1} which corresponds to the overlap of NH and OH vibrational mode. The band around 2900 cm^{-1} was also attributed to the CH asymmetric stretch. The carbonyl and amino vibrational bands around 1637 cm^{-1} and 1527 cm^{-1} correspond to amide I and amide II, respectively. The vibration band around 1238 cm^{-1} was attributed to amide III due to C-N stretching and N-H in-plane bending vibrations [54]. The vibrational bands around 1320 cm^{-1} and 1437 cm^{-1} were attributed to the wagging vibrations of proline side chains confirming the presence of type-I Gel. The FT-IR spectrum of CS/Gel shows all the characteristic functional groups that were present in the spectrum of the individual polymers. The increase in the intensity of the vibrational bands around 1025 cm^{-1} was observed, indicating the interaction between the CS and Gel. Furthermore, the presence of a band around 942 cm^{-1} confirms the interaction between the two polymers. The vibrational band at 942 cm^{-1} was absent from the Gel spectrum and was observed from the CS spectrum at lower energy (891 cm^{-1}). These observations further confirm the interaction between the CS and Gel. The intensity of all the vibrational bands got reduced upon coating with PDA. PDA also have OH and NH functional groups which are also present in CS and Gel structures. These will cause the overlap of these vibrational bands at around 3250 cm^{-1} . The chemical structure of the CS/Gel was maintained after coating. This shows that PDA did not chemically interact with CS/Gel but through hydrogen bonding. The intensity of the vibrational bands decreased further upon incorporation with both Ag and Cu NPs. A slight band shift towards low energies was observed. This could be due to the physical interaction between the NPs and polymers.

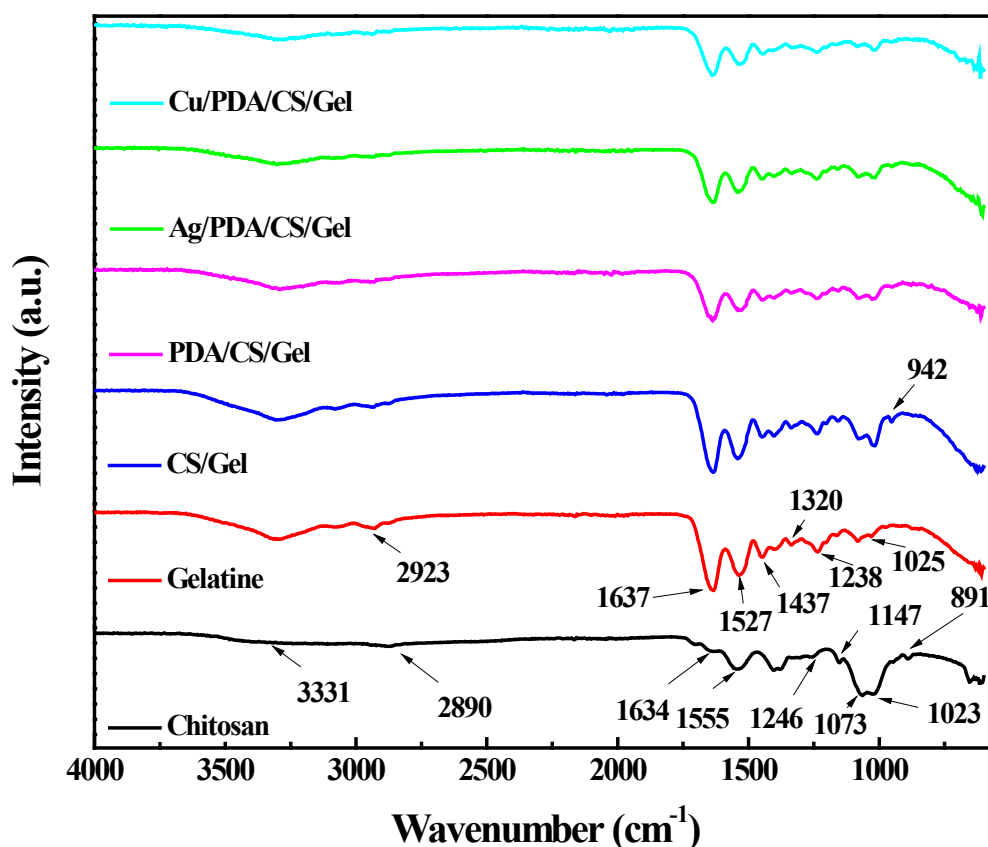


Figure 8.3: FTIR spectra of the synthesized scaffolds, showing their characteristic functional groups.

Three dimensional (3D) scaffolds with good porosity have added advantages in the wound healing process. They can control wound exudates, promote cell migration and guide vascular infiltration, making them suitable dermal substitutes for skin tissue regeneration. To study the scaffolds' morphology/structure and the nature of their pores, SEM was used. Shown in **Figure 8.4** are the SEM micrographs of (a) CS, (b) Gel, (c) CS/Gel and (d) PDA/CS/Gel scaffold. CS showed porous interconnected circular-like 3D structures. Gel depicted irregular flake-like morphology which is also porous and interconnected. The pore sizes of the CS scaffold were between 300 μm and 600 μm . The pore structure of CS was slightly disturbed upon blending with Gel. CS/Gel appeared to have small pores compared to the individual polymers. In addition, the pores of CS/Gel were not well arranged, and the surface looked rougher compared to that of individual CS. The loss in structures and change in the surface texture of individual polymers (CS and Gel) upon blending could be an indication of a successful cross-linking. The structural features of CS/Gel were not affected upon coating with PDA. PDA/CS/Gel scaffold was structurally similar to the CS/Gel scaffold with the pore sizes ranging from 50 μm to 300 μm . The reason PDA coating could not affect the structural

features of CS/Gel could be the fact that the interaction between PDA and CS/Gel was physical. These observations were also made during the FT-IR analysis, wherein introducing PDA in the CS/Gel scaffold did not affect their chemical structure.

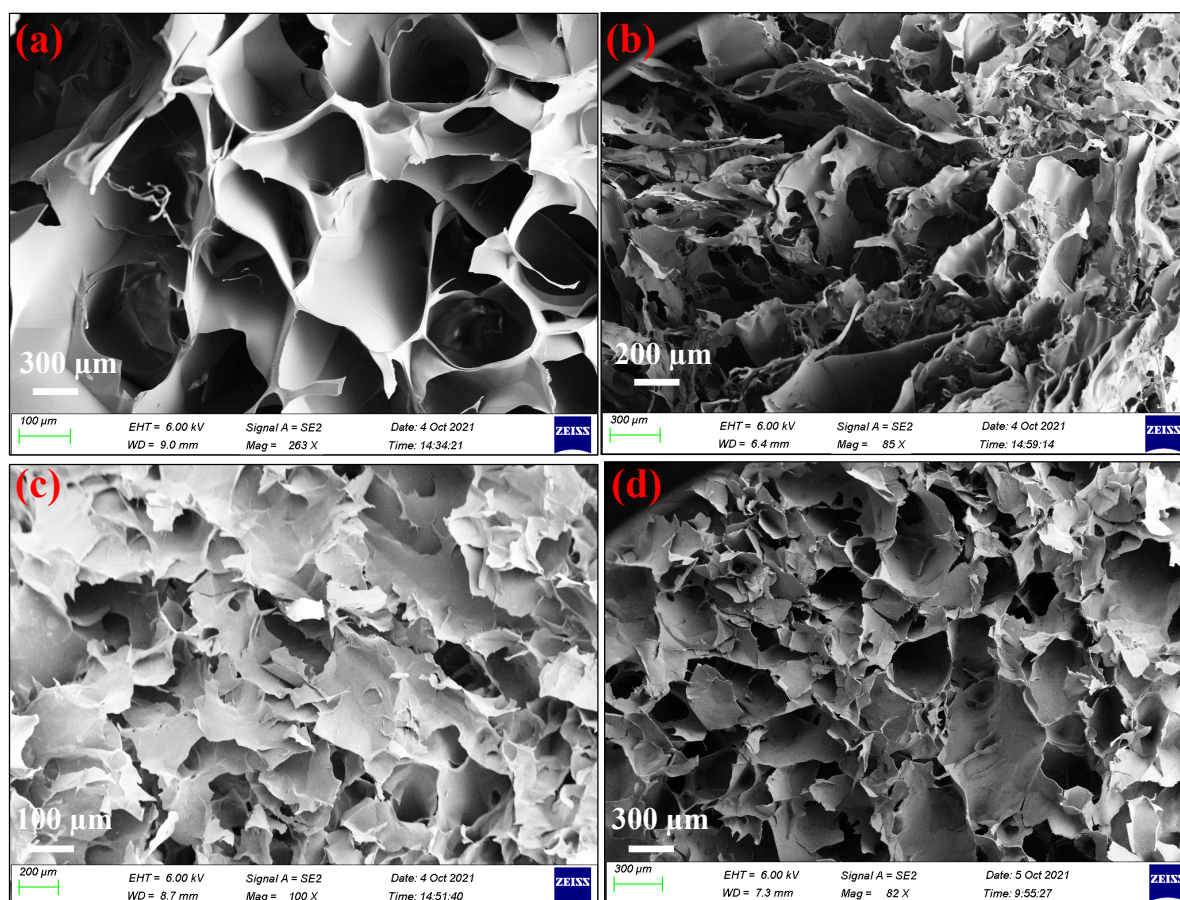


Figure 8.4: SEM micrographs (a) CS, (b) Gel, (c) CS/Gel and (d) PDA/CS/Gel cross-sections.

Figure 8.5(a, c, e and g) shows the SEM micrographs of Ag and Cu NPs decorated scaffolds (Ag@PDA/CS/Gel and Cu@PDA/CS/Gel) prepared with different amounts of metal ions. Their corresponding magnified micrographs are shown in **Figure 8.5(b, d, f and h)**. The incorporation of NPs within the PDA/CS/Gel scaffolds matrix could not disturb their structural features. The pores and the sizes of the decorated scaffolds were still similar to those of PDA/CS/Gel scaffolds. The magnified micrographs revealed the presence of Ag NPs (b and d) and Cu NPs (f and h) in the matrix of the scaffolds. This was further confirmed by energy-dispersive X-ray spectroscopy (EDS) in **Figure 8.6**. These findings prove that PDA successfully coated the surface of the scaffolds. This is because there was no reducing agent used to reduce the Ag ion/salt into metallic Ag NPs. The reduction of Ag ions was facilitated by the presence of catechol and amine functional groups inherent from PDA.

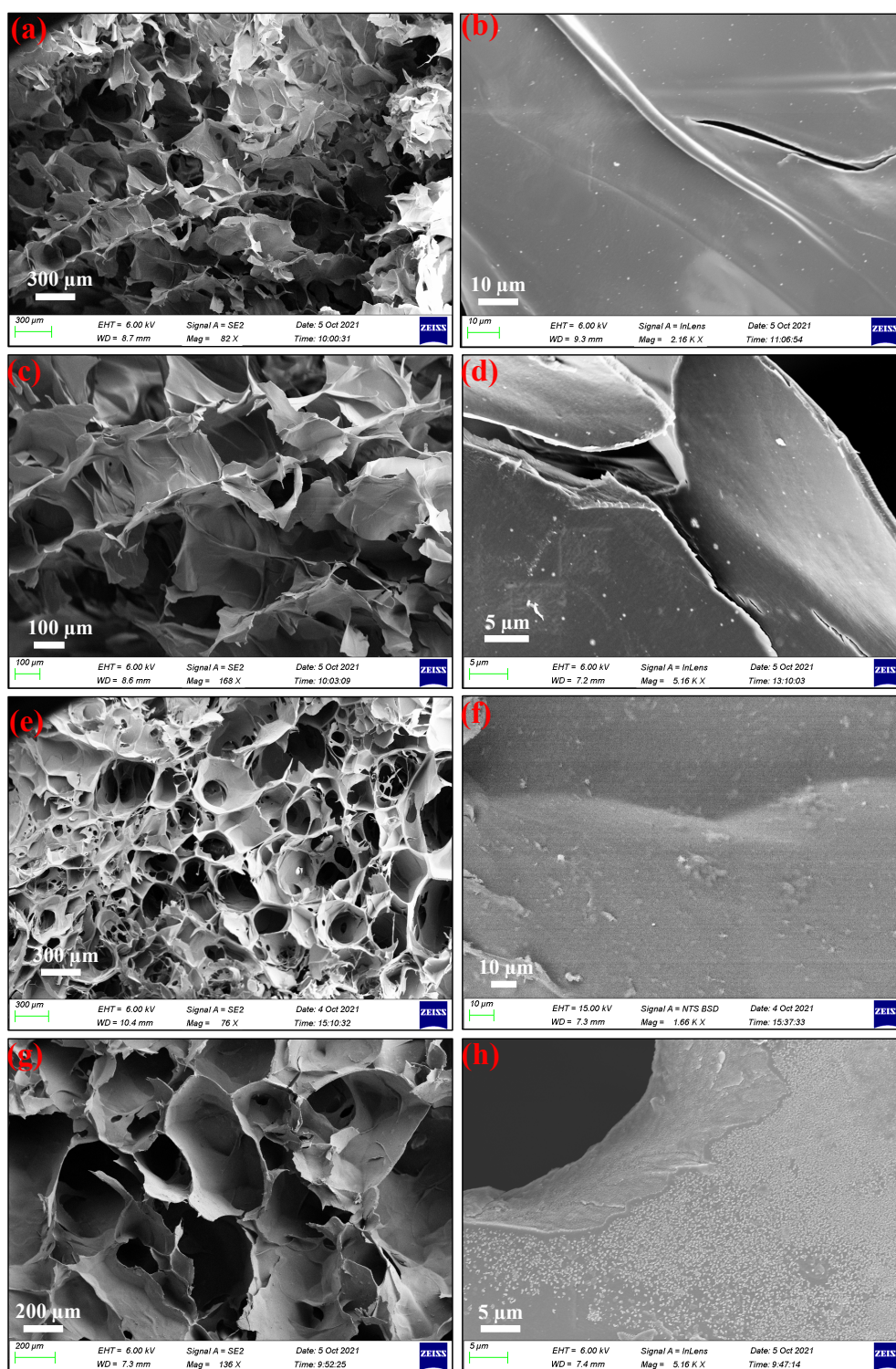


Figure 8.5: SEM micrographs of (a) Ag1@PDA/CS/Gel, (c) Ag2@PDA/CS/Gel and (e) Cu1@PDA/CS/Gel, (h) Cu2@PDA/CS/Gel and (b, d, f, g) their respective magnified micrographs.

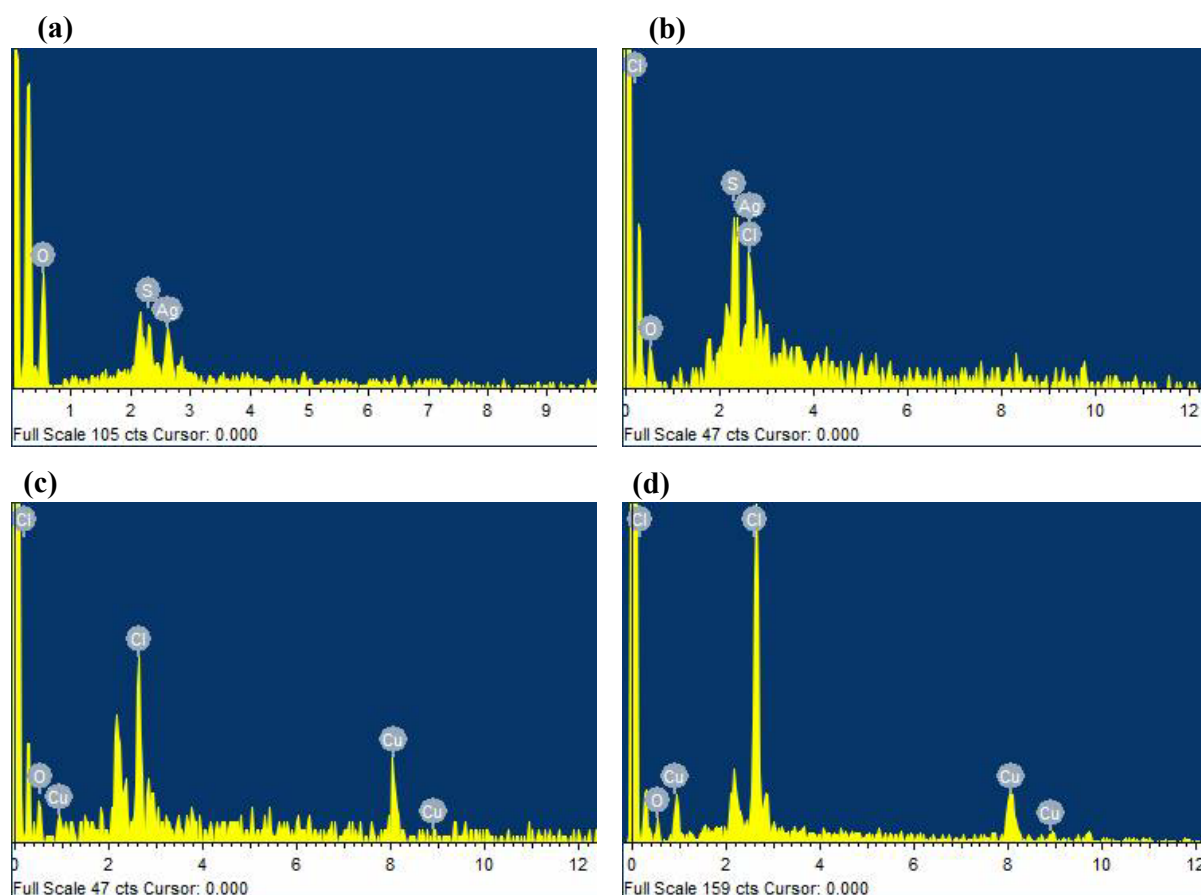


Figure 8.6: EDS spectra of (a) Ag1@PDA/CS/Gel, (b) Ag2@PDA/CS/Gel, (c) Cu1@PDA/CS/Gel and (d) Cu2@PDA/CS/Gel.

The crystallinity and the phase structure of pristine polymers (CS and Gel), CS/Gel, PDA/CS/Gel, Ag@PDA/CS/Gel and Cu@PDA/CS/Gel were elucidated using PXRD. The diffractograms of pristine polymers, CS/Gel and PDA/CS/Gel scaffolds are shown in **Figure 8.7(a)**. A broad diffraction peak was observed between diffraction angles of 10° and 30° from the diffractograms of scaffolds without NPs. This diffraction peak was attributed to the amorphous nature of the polymers. **Figure 8.7** (b and c) respectively show the diffractograms of Ag@PDA/CS/Gel and Cu@PDA/CS/Gel prepared at different concentrations of metal ions. Ag2 and Cu2 were prepared with high concentrations of metal ions as compared to Ag1 and Cu1 based scaffolds. The diffractograms of Ag1@PDA/CS/Gel and Ag2@PDA/CS/Gel are both characterized by two diffraction peaks: a highly intense broad peak between 15° and 25° and the other one at 39° diffraction angle. The broad diffraction peak was attributed to the amorphous nature of the polymers as was also observed from the metal-free scaffolds diffractograms. The diffraction peak at 39° was attributed to the (111) lattice plane of the face centred cubic (FCC) crystal phase of pure metallic Ag (PDF 01-087-0720). The intensity of

the Ag lattice plain was suppressed in both samples. This could be due to the low concentration of the metal ions used relative to that of the polymers, which could also be the reason why other peaks were not present. The (111) plain is densely packed, meaning that it has more scatterers compared to other crystal plains, hence a pronounced peak. The diffractograms of Cu1@PDA/CS/Gel and Cu2@PDA/CS/Gel also displayed two characteristic diffraction peaks. The first broad peak between 15° and 25° was also due to the amorphous nature of polymers. The small and less intense diffraction peak around 43° corresponds to the (111) lattice plane of the FCC crystal phase of metallic Cu (PDF 01-071-4609). Just like in the Ag@PDA/CS/Gel diffractogram, one crystal plain (111) was observed from Cu@PDA/CS/Gel was observed. There were no other impurities observed from both Cu and Ag-based scaffolds. The data obtained from the PXRD analysis confirmed successful *in-situ* incorporation of the NPs within the polymer's matrix. This further confirms that both the Cu and Ag species observed from the EDS were in a metallic rather than ionic state.

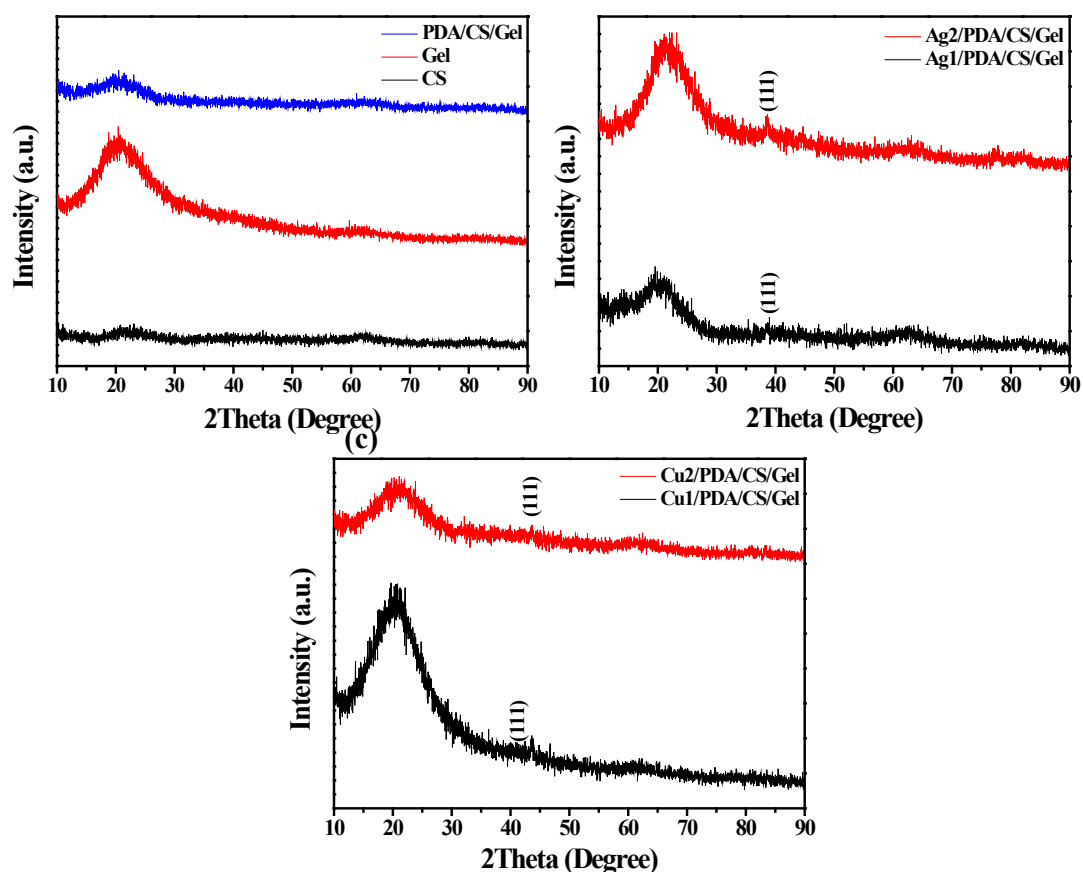


Figure 8.7: PXRD diffractograms of the synthesized scaffolds.

The thermal stability of the scaffolds was conducted using thermal gravimetric analysis (TGA). The TGA analysis was also used to quantify the metal loading within the scaffold's matrix. **Figure 8.8** (a and b) shows the derivative-TGA (D-TGA) and the TGA thermograms of metal-free scaffolds, respectively. The D-TGA thermograms of the metal-free scaffolds displayed various decomposition peaks attributed to multi-step decompositions of the polymers. All the D-TGA thermograms displayed a decomposition peak between 50 °C and 100 °C which are attributed to moisture escapement. The decomposition rate of the scaffolds was relatively slow with Gel possessing the slowest rate. At around 100 °C Gel maintained over 90% of its weight while others were around 80% as shown in the TGA thermograms. A slight increase in the rate of mass loss of CS was observed between 100 °C and 180 °C due to the loss of polymers side functional groups. A gradual increase in the rate of CS weight loss was observed from 250 °C with the decomposition rate peak at 290 °C. This decomposition was due to the breaking of the CS chain. CS started to slowly decompose until it completely lost all its weight at 610 °C because of the polymer backbone and C=C cleavages. The gel was thermally stable until 300 °C where it gradually lost almost 50% of its mass at 380 °C. Just like with CS, the weight loss of Gel was attributed to the decomposition of polymers side functional groups (i.e., OH, NH). Gel also started to decompose slowly until it completely loses its weight around 610 °C due to the complete cleavage of the polymer backbone. The thermal stability of CS/Gel scaffolds was improved compared to that of pristine CS and Gel. The rate of decomposition was reduced, and the scaffolds completely lost their weight at high temperatures (640 °C). The D-TGA and TGA thermograms of PDA/CS/Gel scaffolds appeared to be similar to that of CS/Gel scaffolds. This is an indication that PDA did not affect the thermal stability of the scaffolds nor disturb their chemical composition.

The metal loading on the scaffold's matrix was also investigated from the TGA analysis. **Figures 8.8** (c and e) show the D-TGA while (d and f) show TGA thermograms of Ag@PDA/Gel/CS and Cu@PDA/CS/Gel scaffolds prepared at different amounts of metal ions, respectively. The D-TGA thermograms of the scaffolds displayed the removal of moisture content between 50 °C and 100 °C. Slow decomposition of Ag₁@PDA/CS/Gel scaffolds was observed between 125 °C and 200 °C. Similar observations were also made with Cu₁@PDA/CS/Gel, however, the decomposition rate was slightly higher. A gradual weight loss at a relatively high rate in the range of 250 °C to 350 °C and maximum of around 290 °C was observed for both Ag_{1/2}@PDA/CS/Gel and Cu_{1/2}@PDA/CS/Gel scaffolds. At 500 °C, Ag₂@PDA/CS/Gel scaffolds started to decompose at a faster rate which ended up

losing 87% of its weight at 550 °C. A gradual decomposition of Ag1@PDA/CS/Gel was also observed at 500 °C with 99% of its weight loss at 615 °C, however at a slower rate than Ag2@PDA/CS/Gel. The residue of 1% and 13% remained from Ag1@PDA/CS/Gel and Ag2@PDA/CS/Gel, respectively, were due to the presence of Ag metals in the scaffolds. Cu1@PDA/CS/Gel and Cu2@PDA/CS/Gel scaffolds displayed decomposition steps similar to those from Ag-based scaffolds. This proved that the difference in these scaffolds is the metals, not the polymers. The complete decomposition of the scaffolds was observed at 600 °C for both Cu1@PDA/CS/Gel and Cu2@PDA/CS/Gel with their respective residue of 3% and 21% attributed to the presence of Cu metal. It is important to notice that the Ag and Cu based scaffolds completely lost their weight at relatively lower temperatures than the metal-free scaffolds. This indicates that the presence of metals in the scaffolds reduces the thermal stability of the polymers. This reduction in thermal stability could be attributed to the catalytic nature of the metals which increase the rate of decomposition.

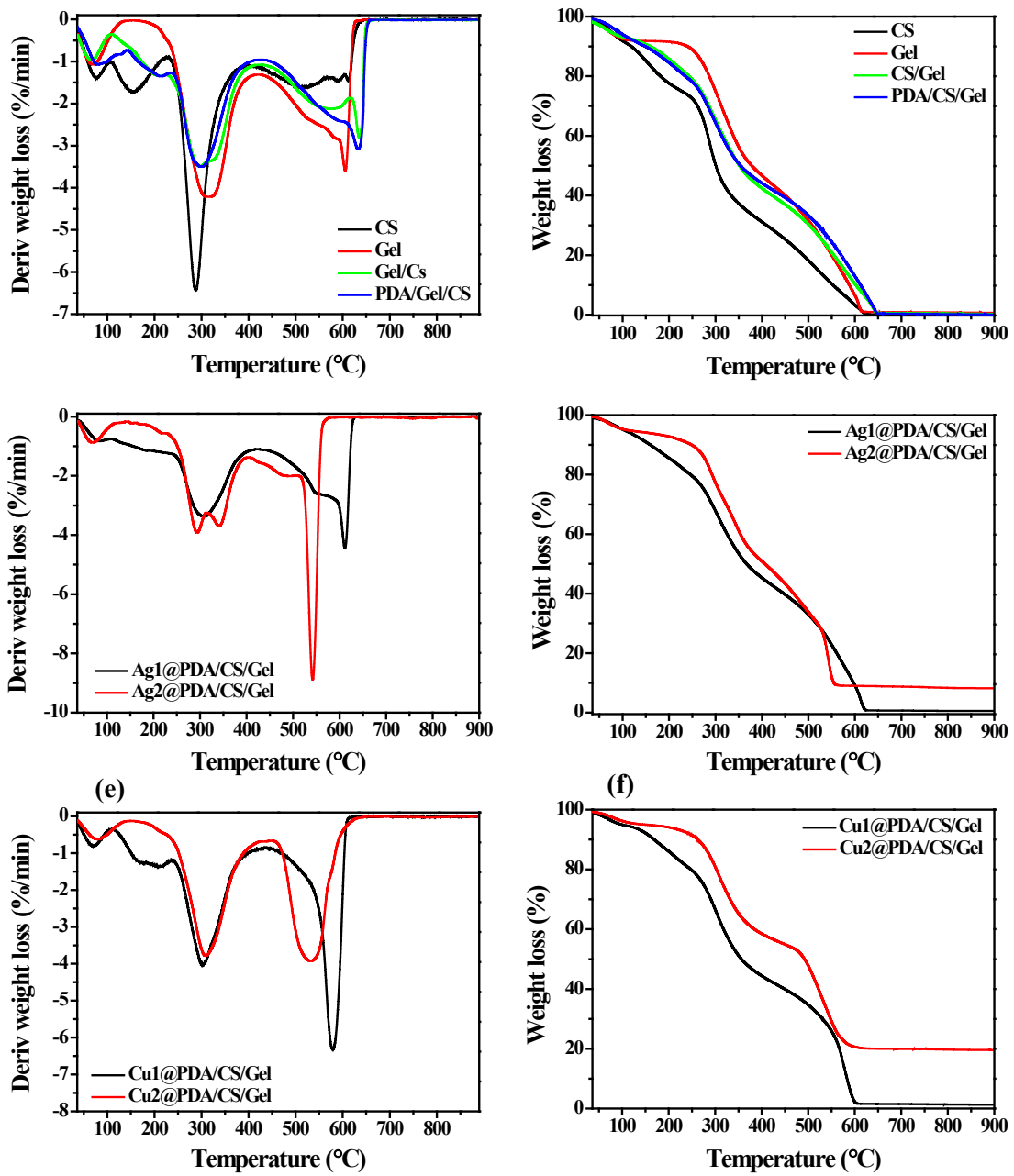


Figure 8.8: The Derivative-TGA of (a) pristine scaffolds, (c) Ag incorporated and (e) Cu incorporated scaffolds together with their corresponding TGAs (b, g, f).

8.3.2 Swelling and Biodegradable studies

The swelling ability and the biodegradability of the scaffolds are very crucial in skin tissue engineering. The perfect scaffold for skin tissue engineering should be able to absorb wound exudate, keep the moist environment, and be able to degrade with time. An added advantage of the skin tissue scaffold would be its ability to unravel bacterial infections since it affects

tissue regeneration and compromises the healing process. The swelling ability was investigated by determining the FU (the amount of fluid the scaffolds absorb) with time. In this case, PBS at pH 7.4 was used as the fluid. The amounts of FU in % form was determined in 20 min intervals for 2 h and the last two values were recorded after 24 h and 48 h and the data are presented in **Figure 8.9a**. All scaffolds showed rapid swelling within the first 20 min. The FU for pristine CS and Gel was relatively low compared to the other scaffolds but continued to increase until 80 min before it plateaued for the rest of the study time. CS showed the least FU, and this can be attributed to the fact that it is insoluble in water. Chitosan requires slightly acidic media ($\text{pH} < 6.5$) to dissolve, and the PBS used in this study had a pH of 7.4. The CS/Gel scaffold showed an improved swelling ability as indicated by the FU of over 1400% within the first 20 min. The FU of CS/Gel remained almost the same as time increased, proving that it had reached equilibrium. The PDA/CS/Gel together with Ag1@PDA/CS/Gel and Cu1@PDA/CS/Gel scaffolds showed similar FU as CS/Gel for the period of the study (2880 min/2 days). The high FU of CS/Gel, PDA/CS/Gel, Ag1@PDA/CS/Gel and Cu1@PDA/CS/Gel scaffolds can be attributed to the crosslinking of the two polymers (CS and Gel) which improved their mechanical properties.

As time passes by, the texture of the crosslinked (CS/Gel) scaffolds changes from being soft sponge-like to a stretch thick hydrocolloid mimicking the texture of skin tissue. This change in texture is very crucial in the wound healing process since it may facilitate the contact of the scaffolds with the skin tissue in the wounded area. The texture of PDA/CS/Gel, Ag1@PDA/CS/Gel and Cu1@PDA/CS/Gel scaffolds did not completely change, or they were rather showing a slow change. This could be attributed to the improved physical properties brought about by coating with PDA. This change in the scaffolds texture when in contact with wound exudates is advantageous in the wound healing process since they can reduce the frequent change of wound dressing or eliminate it, consequently reducing the treatment cost. The other important feature/property of the scaffolds for skin tissue engineering is the ability to maintain their structural integrity and be able to be degraded by body fluids or processes with time. The biodegradability study of the scaffolds was conducted over 7 days and the data is shown in **Figure 8.9b** with the corresponding legend in **Figure 8.9c**. The pristine Gel scaffold appeared to degrade faster than the other scaffolds, which completely degrade within 2 days. Unlike Gel, the pristine CS scaffold showed the least degradation rate compared to other scaffolds. These findings align clearly with the observations made on the FU studies. CS does not have a good swelling ability, which means

it does not have good FU hence a slow degradation. The crosslinked CS/Gel scaffold on the other hand showed a low biodegradation rate compared to Gel, but a fast one compared to CS. The rate of biodegradation of the crosslinked CS/Gel started to slow down after 2 days with 30% of the content remaining. The slow biodegradation continued for the duration of the study with the biodegradation of almost 80% after 7 days. This controlled biodegradation of CS/Gel with time was anticipated due to crosslinking that improves the mechanical strength of the scaffolds. Previous studies have also shown the importance of polymer and protein crosslinking in improving the biodegradability and the mechanical properties of the scaffolds to be applied in tissue engineering. The biodegradability of PDA coated, and NPs incorporated scaffolds (PDA/CS/Gel and Ag@PDA/CS/Gel) appeared to be increasing with time in a well-controlled manner compared to the rest of the scaffolds. This indicates the ability of PDA to control the biodegradability of the scaffolds with time, which is highly recommended in skin tissue engineering. Moreover, these scaffolds managed to maintain almost perfect structural integrity during the degradation period, which may help in terms of controlling the wound exudate while keeping a moist environment and providing a barrier against infection. However, the CuI@PDA/CS/Gel scaffold was found to degrade quicker than anticipated. The fast degradation of this scaffold could be attributed to the presence of reducing agent (hydrazine hydrate) that was used to reduce Cu salt during the formation of Cu NPs. Hydrazine hydrate could have cleaved the crosslinking bonds between genipin and the two polymers (CS and Gel) which could affect the mechanical strength of the scaffold, hence fast degradation.

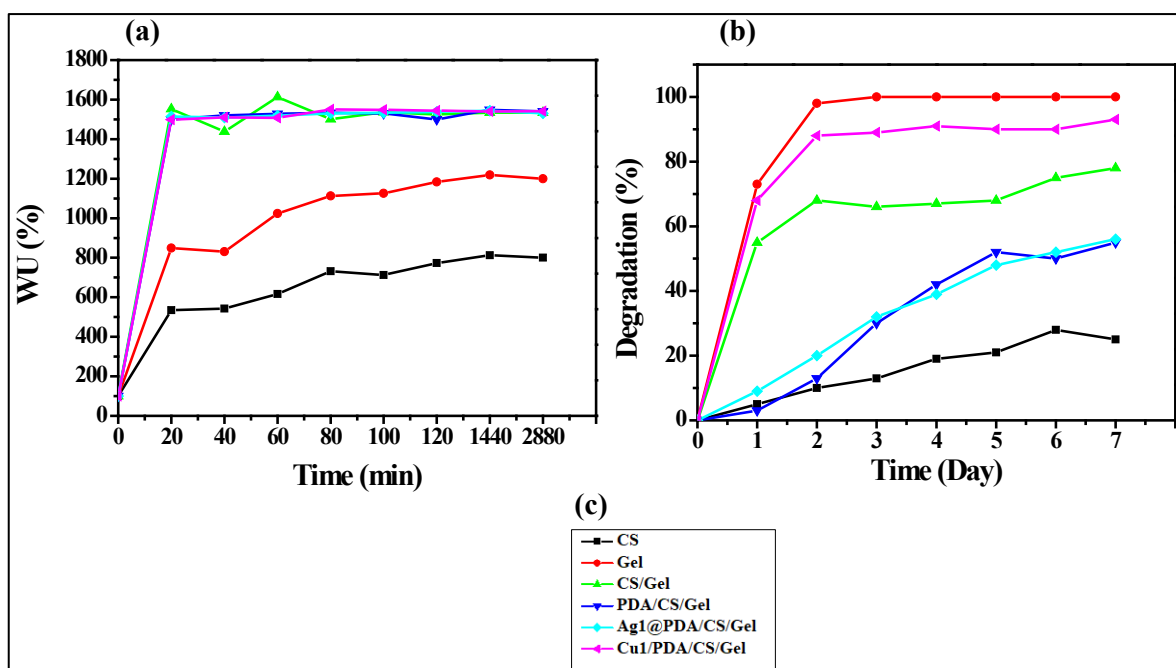


Figure 8.9: Graphs showing (a) the FU and (b) the biodegradability of the scaffolds.

8.3.3 Antimicrobial activity of the scaffolds

The antimicrobial activity of the scaffolds was investigated against gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria using a standard well-diffusion method. The ability of the scaffolds to inhibit bacterial growth was assessed by observing the clear zones of bacterial inhibition on the agar plates. **Figures 8.10** (a and b) show the photographs of agar plates with the bacterial inhibition zones due to pristine and Ag-based scaffolds while Figures 7c and d show bacterial inhibition zones due to pristine and Cu-based scaffolds against *E. coli* and *S. aureus*. All the scaffolds showed to have a certain level of antimicrobial activity. The antimicrobial activity of CS and Gel was very low as indicated by small zones of inhibition. It appears that crosslinking the two polymers and coating them could not have any significant effect on the antimicrobial activity of the polymers. This can be seen by more or less the same sizes of zones of inhibition of CS, Gel, CS/Gel and PDA/CS/Gel scaffolds which were found to be between 9 mm and 10 mm against both strains as shown in table 1. The minimum inhibitory concentrations (MIC) of these scaffolds were also found to be the same (150 µg/ml). The MICs of these scaffolds were determined to investigate their antimicrobial activity, quantitatively, through the serial dilution method and are also shown in table 1. Incorporating Ag and Cu NPs on the surface of the scaffolds have significantly improved their antimicrobial activity. This is due to the wide spectrum of antimicrobial activity that Ag and Cu NPs possess. Incorporating these NPs with the scaffolds brings about a synergy effect

towards antimicrobial activity. The zones of inhibition of both Ag and Cu NPs incorporated scaffolds appeared too big compared to the other scaffolds. The zones of inhibition became even bigger when the concentrations of Ag or Cu salt was increased. This can be seen from the zones labelled F, in **Figure 8.10**, which represents either Ag₂@PDA/CS/Gel (**Figure 8.10a** and **b**) or Cu₂@PDA/CS/Gel (**Figure 8.10c** and **d**). The improved antimicrobial activity of the scaffolds due to NPs incorporation was further supported by the values of MIC obtained as shown in table 1. The MIC values decreased from 150 µg/ml to 75 µg/ml for Ag₁@PDA/CS/Gel and to 37.5 µg/ml for Ag₂@PDA/CS/Gel. Cu₁@PDA/CS/Gel did not show any decrease in MIC compared to other scaffolds, however, the MIC of Cu₂@PDA/CS/Gel was found to be 75 µg/ml. This decrease in MIC values is an indication of an improved antimicrobial activity of the scaffolds, which means that compared to other scaffolds, only a few number of NPs incorporated scaffold is required to inhibit bacterial growth.

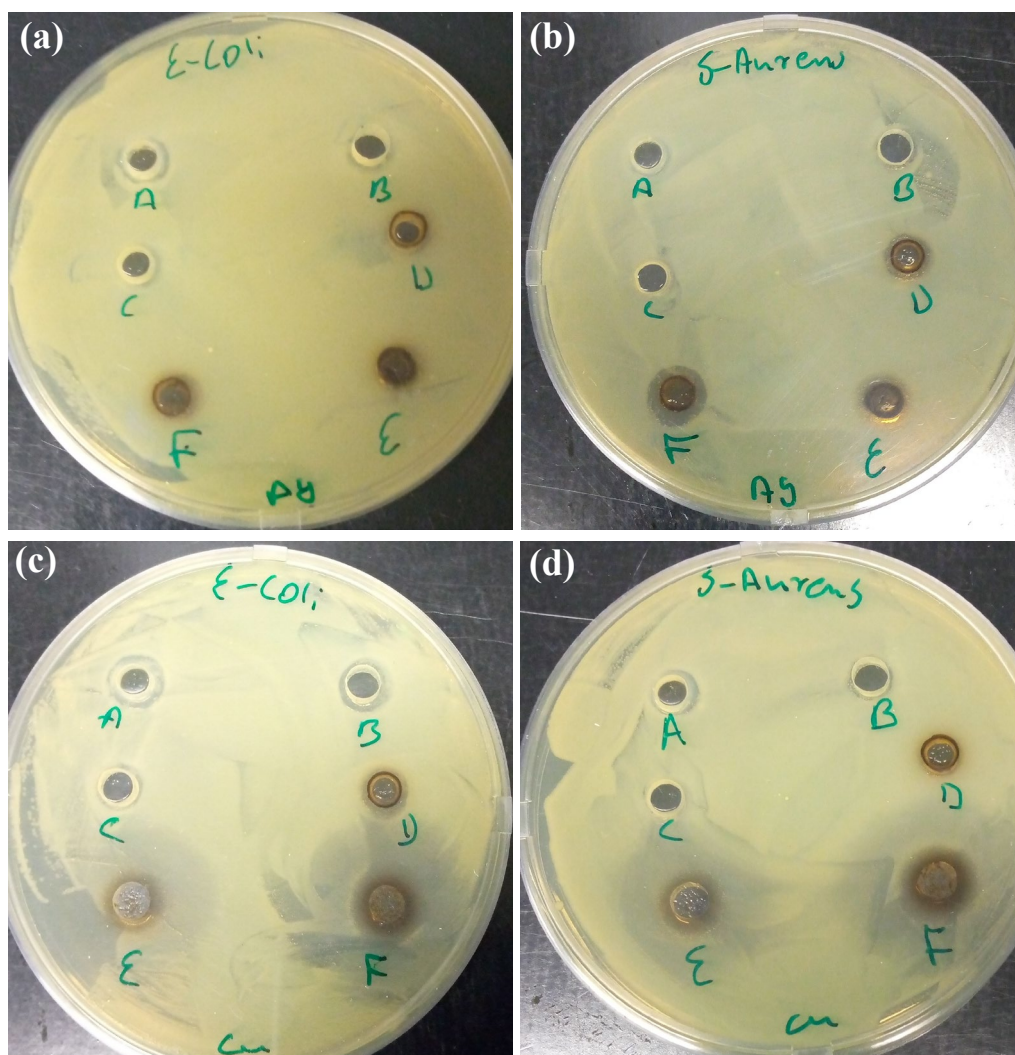


Figure 8.10: Antimicrobial activity of (a, b) Ag and (c, d) Cu based scaffolds against *E. coli* and *S. aureus*. [(A) CS, (B) Gel, (C) CS/Gel, (D) PDA/CS/Gel, (E) Ag1@PDA/CS/Gel and Cu1@PDA/CS/Gel, (F) Ag2@PDA/CS/Gel and Cu2@PDA/CS/Gel].

Table 8.1: Zones of inhibition and the MIC values of the scaffolds against *E. coli* and *S. aureus*. [(A) CS, (B) Gel, (C) CS/Gel, (D) PDA/CS/Gel, (E) Ag1@PDA/CS/Gel and Cu1@PDA/CS/Gel, (F) Ag2@PDA/CS/Gel and Cu2@PDA/CS/Gel]

Sample	Zone of Inhibition (mm)				MIC ($\mu\text{g/ml}$)			
	Ag-based scaffolds		Cu-based scaffold		Ag-based scaffolds		Cu-based scaffolds	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
A	9	8	9	9	150	150	150	150
B	9	8	8	9	-	-	-	-
C	8	7	8	8	150	150	150	150
D	9	9	9	8	150	150	150	150
E	11	10	10	11	75	75	150	150
F	12	14	14	12	37.5	37.5	75	75

8.3.4 Biocompatibility studies

The *in vitro* biocompatibility of the scaffolds was conducted on the BHK-21 (fibroblasts) cell line by MTS proliferation assay. Fibroblasts play a vital role during wound healing process. They play a huge factor in deposition of ECM and generation of response to endogenous stimuli such as growth factor. They are also responsible in the synthesis of collagen. Therefore, understanding the biocompatibility or cytotoxicity of the scaffolds towards such cells is crucial as far as wound healing is concerned. The biocompatibility studies showed that exposing the scaffolds to the BHK-21 cells does not have a negative impact on their viability. As shown in **Figure 8.11**, the cell viabilities of all the scaffolds were comparable to that of control (i.e., untreated cells). This is an indication that the scaffolds were not toxic to the cells and are suitable for skin tissue repair. To further investigate the biocompatibility of the scaffolds and their ability to be used in skin tissue repair, cell adhesion study was conducted. In this study, cells were cultured on the surface of the scaffolds. **Figure 8.12** shows the optical images of the cultured cells with (a) being the control culture (untreated) and (b-e) scaffolds cultures after (A) 24 and (B) 48 h. A uniform growth and morphology of fibroblasts was observed from untreated, and scaffolds treated cultures after 24 h. Cell adhesion within the matrix of scaffolds was also observed. Cells treated with scaffolds showed a rapid growth compared to untreated cells within 24 h of culture. This is an indication that the scaffolds have ability to influence cell proliferation. After 48 h of culture,

the cells appeared to have grown further compared to 24 h for both untreated and scaffolds treated. These results could explain the excellent swelling ability of the scaffolds. Moreover, ability of these scaffolds to influence proliferation while maintaining the fibroblasts morphology makes them an excellent candidate for skin tissue repair.

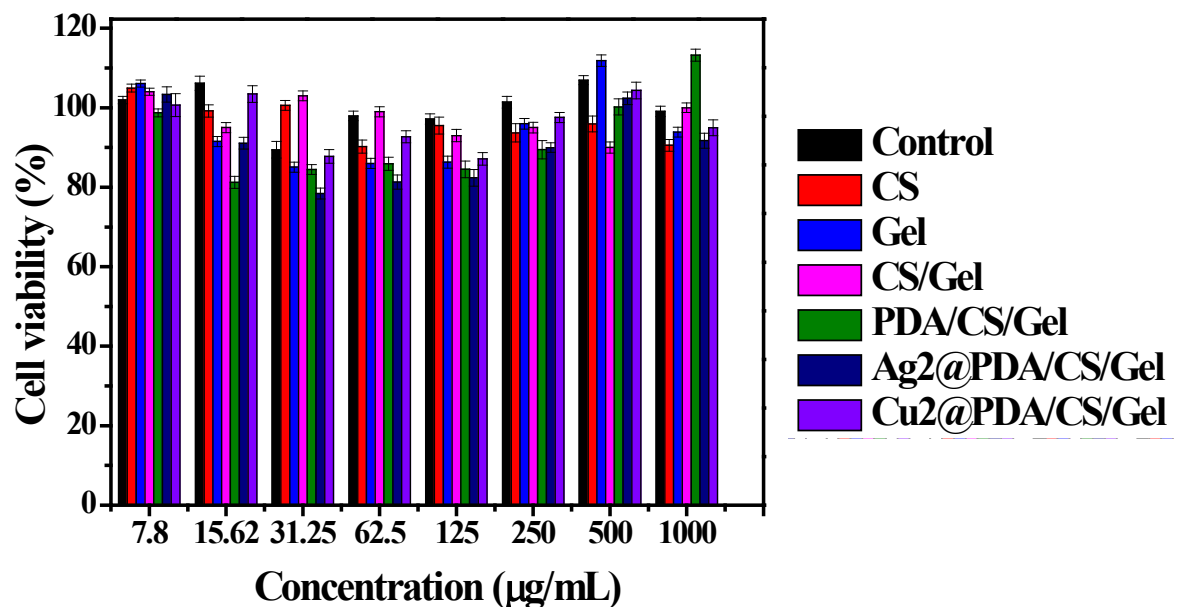


Figure 8.11: Cell viability studies of the scaffolds towards BHK-21 cells.

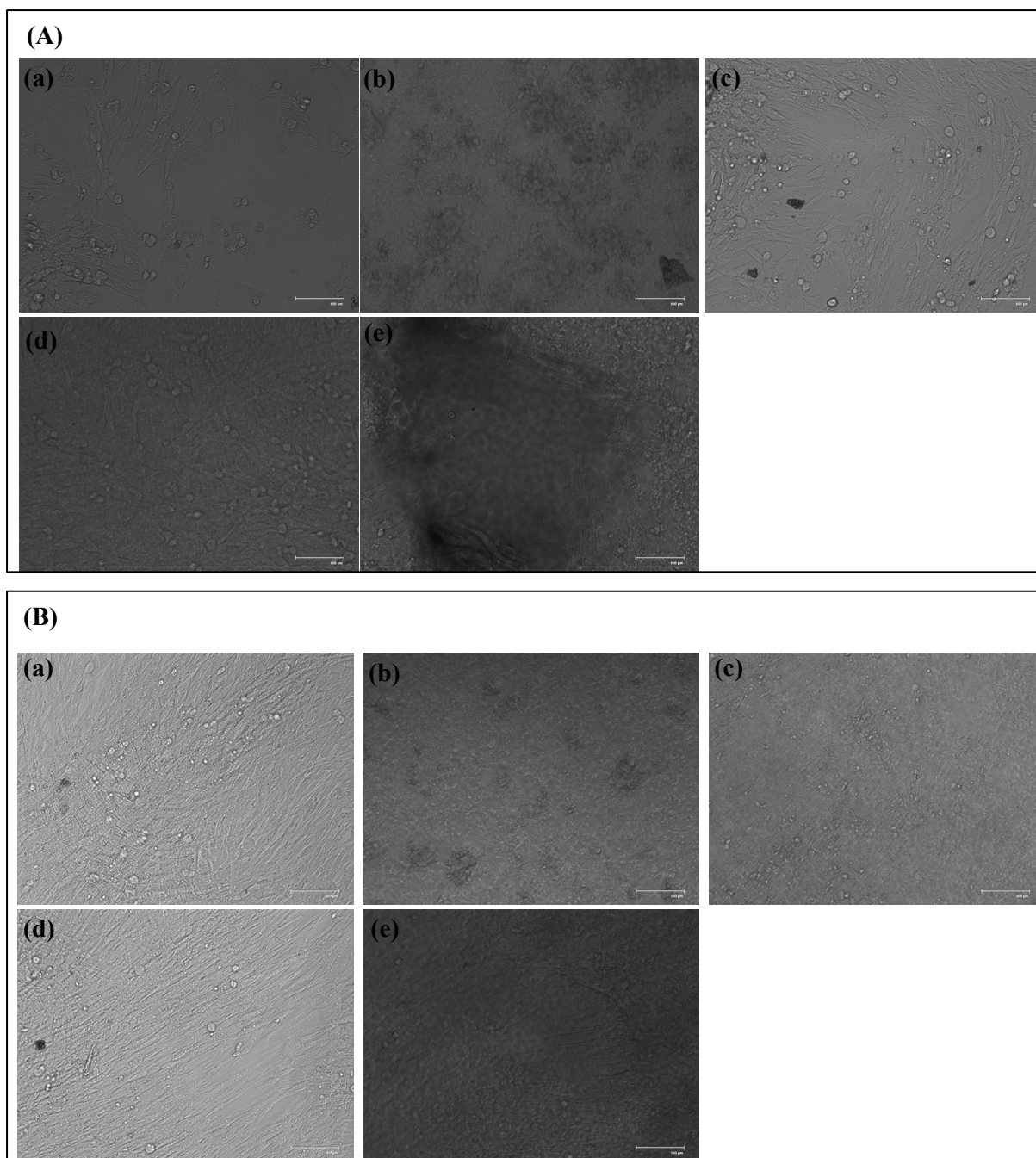


Figure 8.12: Optical microscopy images (scale bar = 100 μm) of (a) untreated fibroblast (BHK-21) cells (control), (b) CS/Gel, (c) PDA/CS/Gel, (d) Ag₂@PDA/CS/Gel and (e) Cu₂@PDA/CS/Gel treated fibroblasts.

8.4 Conclusions

In this study, bioactive materials with the potential to be applied in wound healing were successfully prepared through incorporation of Ag and Cu NPs on PDA coated CS/Gel scaffolds. The crosslinking of CS and Gel was achieved by using genipin which was confirmed by a change in colour (from white to light pink) and supported by chemical

analysis using FTIR. The coating of CS/Gel scaffolds with PDA was confirmed by the change in colour (from light pink to black) which was also supported by FTIR analysis. The morphology studies through SEM demonstrated the porous nature of scaffolds. SEM results supported by EDS, revealed the presence of Ag and Cu NPs within the matrix of the scaffolds. The structural analysis conducted using PXRD further confirmed the *in situ* reduction of Ag and Cu ions to their zerovalent/ metallic states. The results further revealed that the scaffolds were produced with excellent degree of swelling, with CS/Gel, PDA/CS/Gel, Ag@PDA/CS/Gel and Cu@PDA/CS/Gel having comparable and higher swelling ability than pristine CS and Gel. These should ensure effective wound exudate management, which is one of the key factors wound management. The scaffolds endowed biodegradable properties with excellent antimicrobial activity against gram-negative and gram-positive bacterial strains. Moreover, the scaffolds demonstrated appropriate biocompatibility with good cell adhesion on their surfaces. Therefore, these novel scaffolds, should accelerate the wound healing process since they possess relevant properties wound healing properties. Future research on the properties of these scaffolds will be conducted to ensure the optimal wound healing potential.

8.5 References

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Chapter 9

General conclusions and recommendations

9.1 General conclusions

This thesis reports on the fabrication of bioactive materials based on Ag and Cu NPs incorporated on biodegradable polymeric scaffolds for potential application in wound healing studies. The study demonstrated facile methods for synthesizing pristine NPs (Ag and Cu), nanocomposites and scaffolds materials. The antimicrobial activity of these materials was investigated against gram-negative and gram-positive bacterial strains. The cytotoxicity of the materials towards BHK-21 fibroblasts was also investigated to assess their biocompatibility at their active concentrations (i.e., MIC values). Firstly, this thesis showed the colloidal synthesis of AgNPs using DA as a reducing agent for the first time. The sizes of the NPs were varied by changing the concentration of the AgNO₃, which was proven by TEM analysis. The successful synthesis of these NPs was further confirmed by PXRD analysis. The NPs exhibited great antimicrobial activity against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacterial strains with negligible toxicity toward BHK-21 cells. The NPs showed a size-dependent antimicrobial activity, with smaller NPs proving to be more potent than bigger ones. The thesis further reported on a facile synthesis of AgNPs coated with PDA (Ag-PDA) and compared their bioactivity with AgNPs incorporated on the surface of PDA spheres (Ag@PDA). Both nanocomposites possessed excellent antimicrobial activity with low to non-toxicity at moderated concentrations. However, the antimicrobial activity of Ag@PDA was higher compared to Ag-PDA, which was attributed to the ease of NPs to interact with the bacteria since they were on the outside surface of PDA, thus more exposure. At high concentrations, Ag@PDA became more toxic than Ag-PDA, which was also attributed to the exposure of the NPs to the cells. Regardless of their excellent antimicrobial activity, Ag@PDA nanocomposites were toxic at high concentrations compared to Ag-PDA, however this could not limit these materials for application in wound healing and other related studies since only a minute concentration is needed for their antimicrobial activity.

The synthesis, antimicrobial activity and cytotoxicity of Cu-based bioactive materials were also investigated. Cu, Cu₂O and the mixture of Cu and Cu₂O (Cu/Cu₂O) NPs were synthesized with hydrazine and DA as reducing and capping agents, respectively. The NPs with spherical shapes were obtained evident from TEM analysis. PXRD and XPS studies

confirmed the phases, chemical composition and oxidation states of the NPs. The antimicrobial activity of the NPs was also investigated against *E. coli* and *S. aureus*. Cu and Cu₂O exhibited promising antimicrobial activity. The Cu/Cu₂O NPs showed an improved antimicrobial activity compared to Cu and Cu₂O NPs due to the synergistic effect. The cytotoxicity of the NPs towards BHK-21 was also evaluated. Cu and Cu/Cu₂O NPs were found to be more biocompatible compared to Cu₂O NPs. Incorporating Cu NPs on the surface of PDA (Cu@PDA) or within the PDA matrix (Cu-PDA) displayed an excellent antimicrobial activity and reduced cytotoxicity compared to pristine Cu NPs. Cu@PDA exhibited strong antimicrobial activity compared to Cu-PDA. The nanocomposites were biocompatible, showing cell viability of over 80 % at high concentrations (i.e., above 500 µg/mL).

A novel and facile synthesis route for the fabrication of DA capped Cu/Ag bimetallic nanoalloys was also shown in this thesis. The bimetallic nanoalloys with fibrous morphology were obtained evident from TEM analysis. PXRD studies confirmed the successful synthesis of the nanoalloys. The effect of Cu:Ag mole ratio on their morphology, antimicrobial activity and cytotoxicity was investigated. Pure fibres were obtained at a 1:1 Cu:Ag mole ratio (BNF-1). Few spherical NPs were observed when 2:1 (BNF-2) and 1:2 (BNF-3) mole ratios were used. BNF-3 possessed strong antimicrobial activity compared to BNF-1 and BNF-2. BNF-1 appeared to be less toxic than BNF-2 and BNF-3. However, a gradual decrease in cell viability was observed as the concentration increased.

Conclusively, this thesis showcased a simple route of incorporating Ag and Cu NPs on the surface of biodegradable PDA coated CS/Gel scaffolds. The *in situ* reduction of Ag ions was achieved without using an additional reductant due to the presence of PDA functional groups during the synthesis of Ag@PDA/CS/Gel scaffolds. An additional reducing agent was needed to incorporate Cu@PDA/CS/Gel scaffolds since PDA functional groups could not ultimately reduce Cu ions independently. The scaffolds with porous matrix were obtained, as was evident from SEM analysis. EDS and PXRD confirmed the successful synthesis and incorporation of pure metallic Ag and Cu NPs on the surface of the scaffolds. TGA analysis further confirmed the presence of NPs as was seen from residual remaining after the polymer burnt out completely. The swelling and biodegradable studies indicated that the scaffolds could easily absorb fluids while degrading with time. The FU uptake by CS and Gel was relatively low (500 % and 1000 %, respectively) compared to PDA/CS/Gel (1500 %). The incorporation of NPs on the PDA/CS/Gel scaffolds did not impact the FU of the scaffolds.

Biodegradable studies showed that the scaffolds could degrade with time when exposed to biofluids.

An excellent antimicrobial activity of the scaffolds was observed against *E. coli* and *S. aureus*. NPs incorporated scaffolds possessed intense activity against both bacterial strains compared to the pristine CS, Gel and PDA/Gel/CS. The MIC data revealed that Ag@PDA/CS/Gel scaffolds exerted strong antimicrobial activity than Cu@PDA/CS/Gel scaffolds. The biocompatibility study of the scaffolds was conducted through their cytotoxicity towards fibroblasts cells (BHK-21). All the designed scaffolds were biocompatible with very low toxicity towards the cells. All the scaffolds had cell viability of over 85%, even at a concentration as high as 1000 µg/mL. The scaffolds also appeared to promote the adhesion and proliferation of the cells. Cell adhesion and proliferation were seen to be improving with time. There was no sign of change in the morphology of the cells due to the presence of the scaffolds. In general, the excellent FU uptake, biodegradability, biocompatibility, antimicrobial activity and the ability to promote cell adhesion and proliferation make these scaffolds the best candidate for skin tissue engineering. This is because they will be able to control exudates and infection in wounds while promoting skin tissue regeneration which is advantageous for skin substitutes.

9.2 Recommendations

For materials to be used in wound healing applications, it is essential to understand their properties beyond antimicrobial activity and cytotoxicity. Although the antimicrobial activity and cytotoxicity of the materials synthesized in this study were conducted, more work on other parameters still has to be shown to validate them used in wound dressings or as skin substitutes. Therefore, the future work listed below is recommended in order to fulfil the requirements of the materials to be used as wound dressings ingredients and skin substitutes. This project has received funding to be carried on to the post-doctorate level and a seed funding for prototype development.

- Optimization of the NPs and nanocomposites concentrations to avoid toxicity towards normal cells
- Antimicrobial studies against strains not used in this study, including MDR bacteria and fungi.
- Optimization of NPs loading on the surface of the scaffolds.
- Investigation of the scaffold's mechanical properties and their antioxidant activity.
- In-vitro wound healing investigation through cell migration/ scratch assay studies.

- In-vivo wound healing studies using animal models
- Materials optimization and development of a working prototype.

Appendix

Chapter 3: Supplementary materials

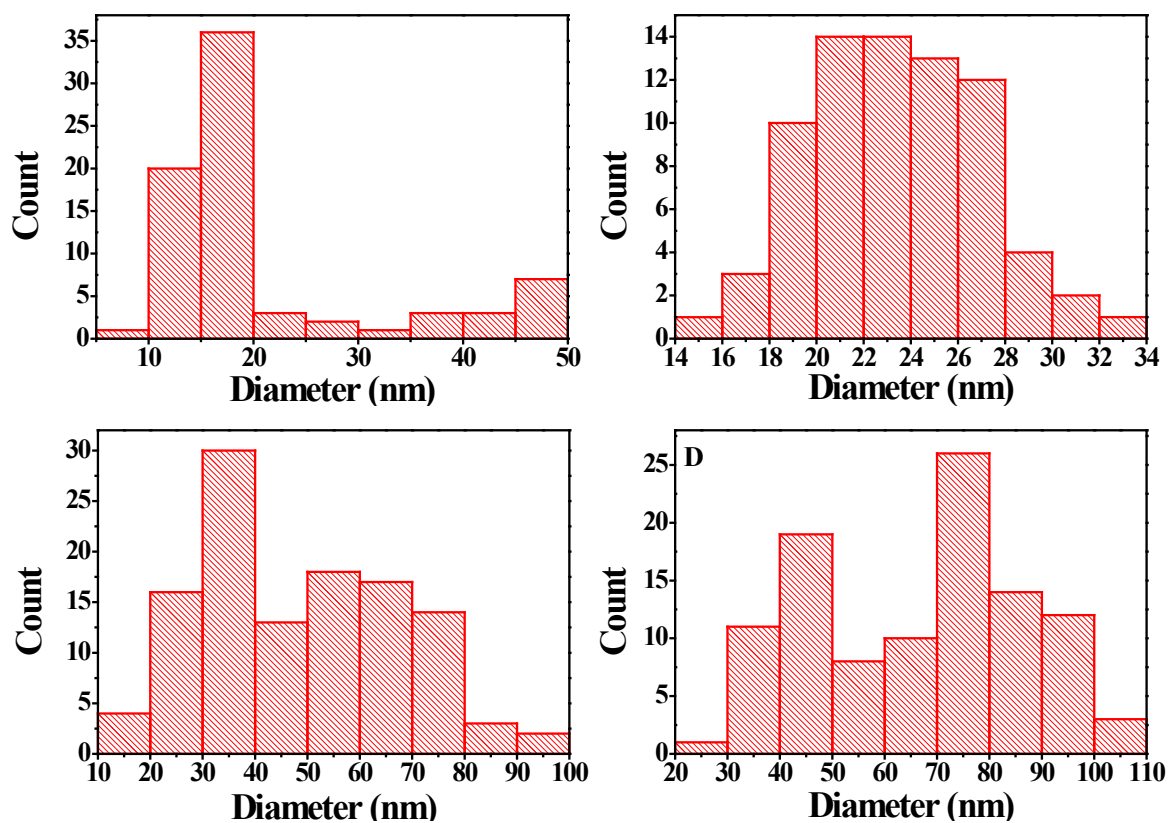


Figure S3.1: Particle size distribution of synthesized AgNPs-DA with the average diameter of (A) $16 \text{ nm} \pm 6 \text{ nm}$, (B) $24 \text{ nm} \pm 2.1 \text{ nm}$, (C) $48 \text{ nm} \pm 3.2 \text{ nm}$ and (D) $74 \text{ nm} \pm 2.8 \text{ nm}$.

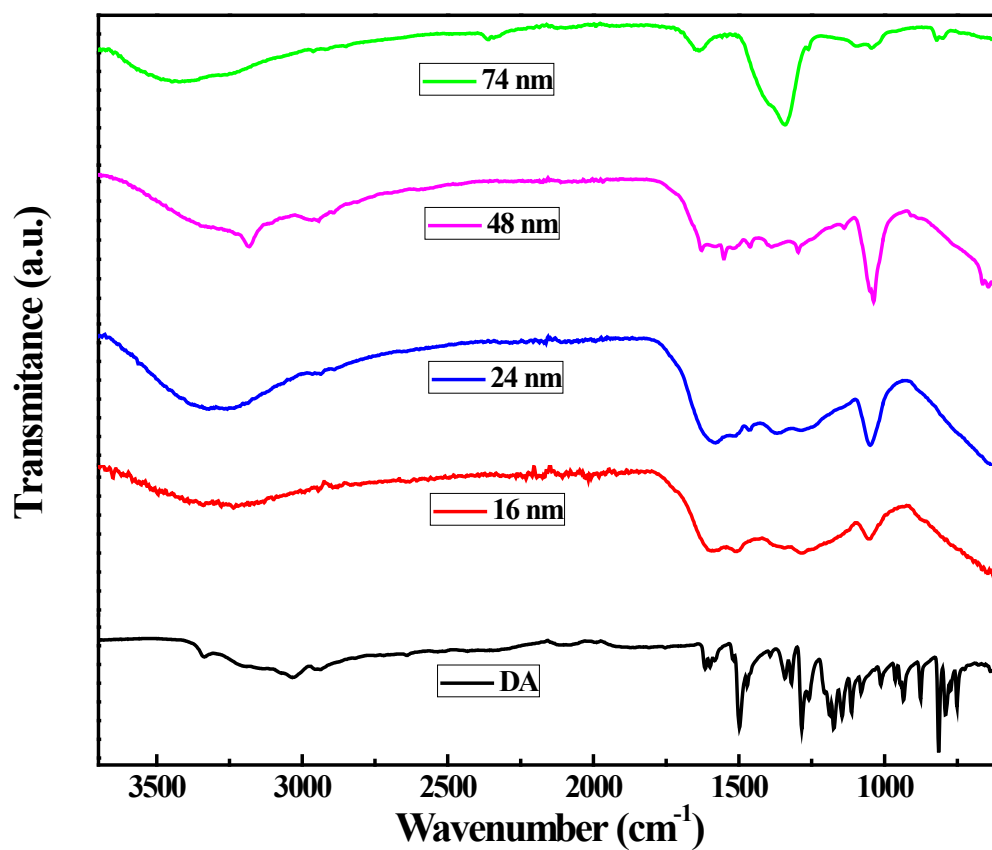


Figure S3.2: The FTIR spectra of the synthesized AgNPs-DA.

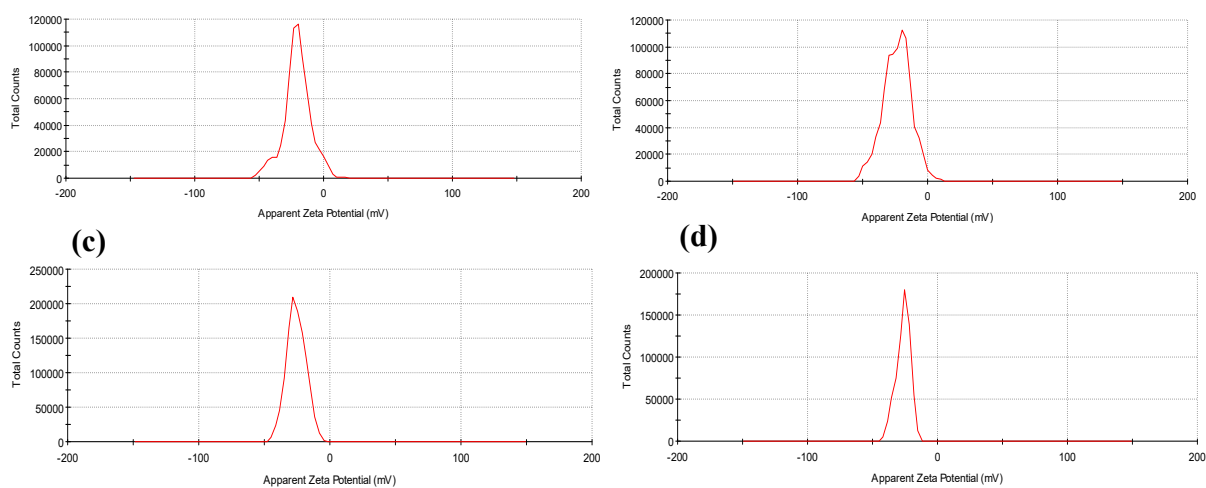


Figure S3.3: Plots of zeta potential of the synthesized AgNPs-DA with the average diameter of (A) 16 nm, (B) 24 nm, (C) 48 nm and (D) 74 nm.

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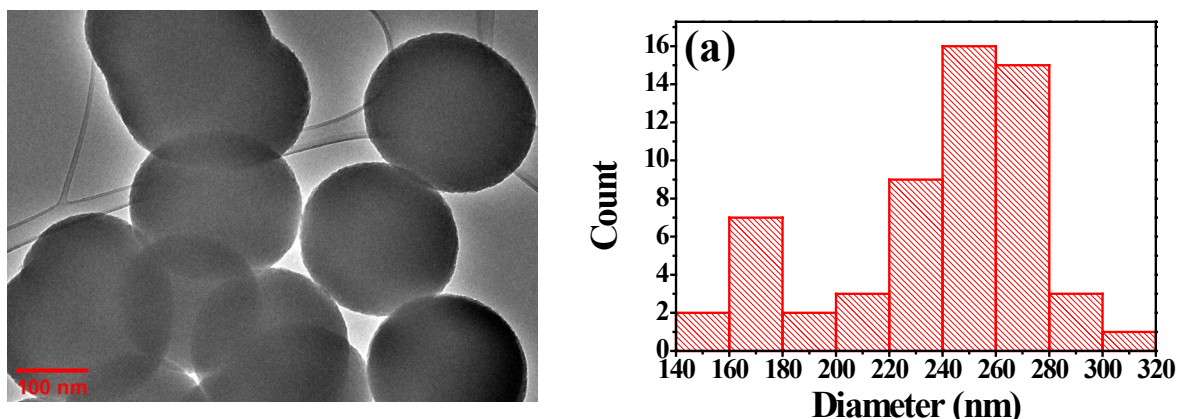


Figure S4.2: TEM micrograph (a) and particle size distribution (b) of PDA spheres

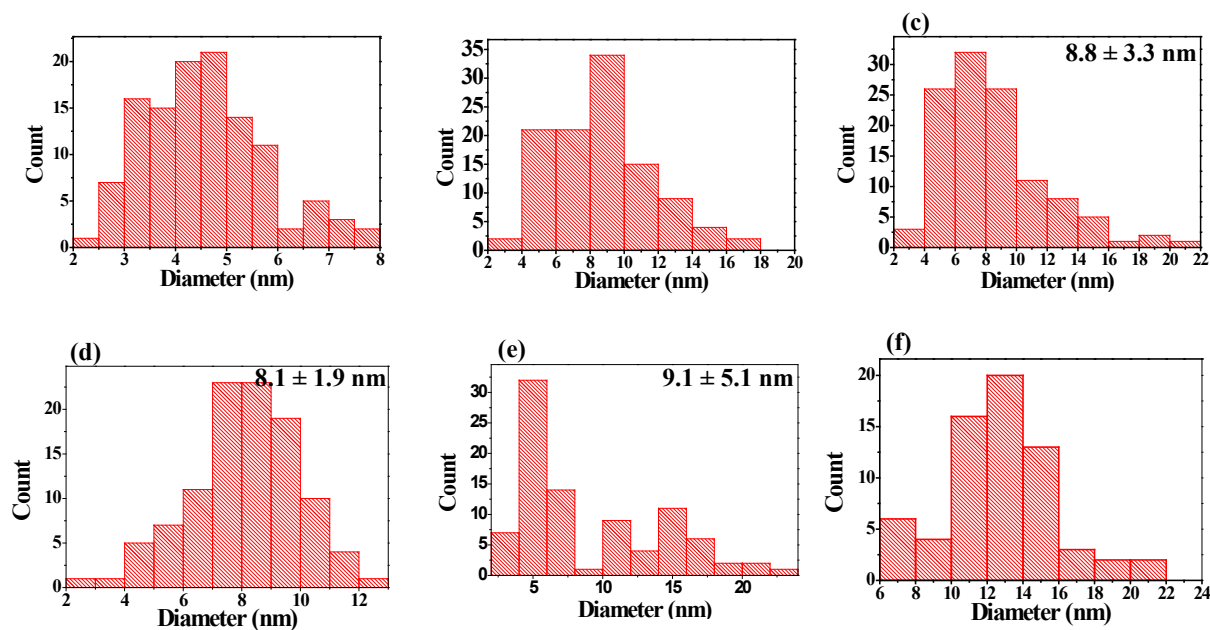


Figure S4.3: Particle size distributions of (a-c) Ag-PDA and (d-f) Ag@PDA nanocomposites at different AgNO_3 concentrations. [(a, d) 1 mM, (b, e) 5 mM and (c, f) 10 mM].

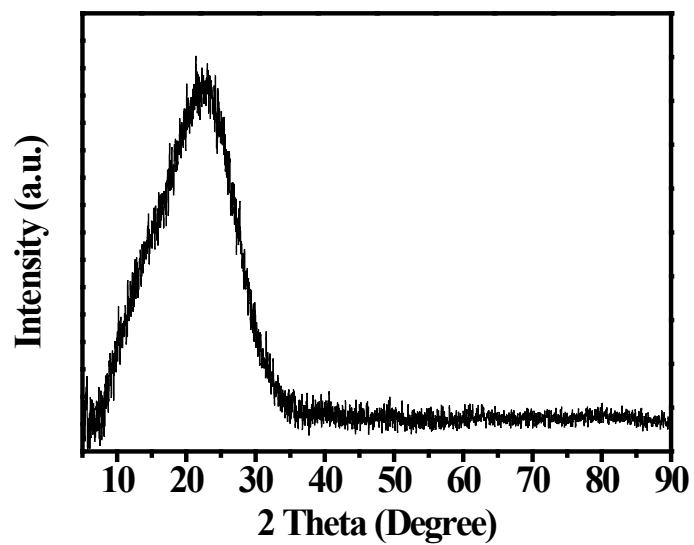


Figure S4.4: PXRD pattern of PDA spheres

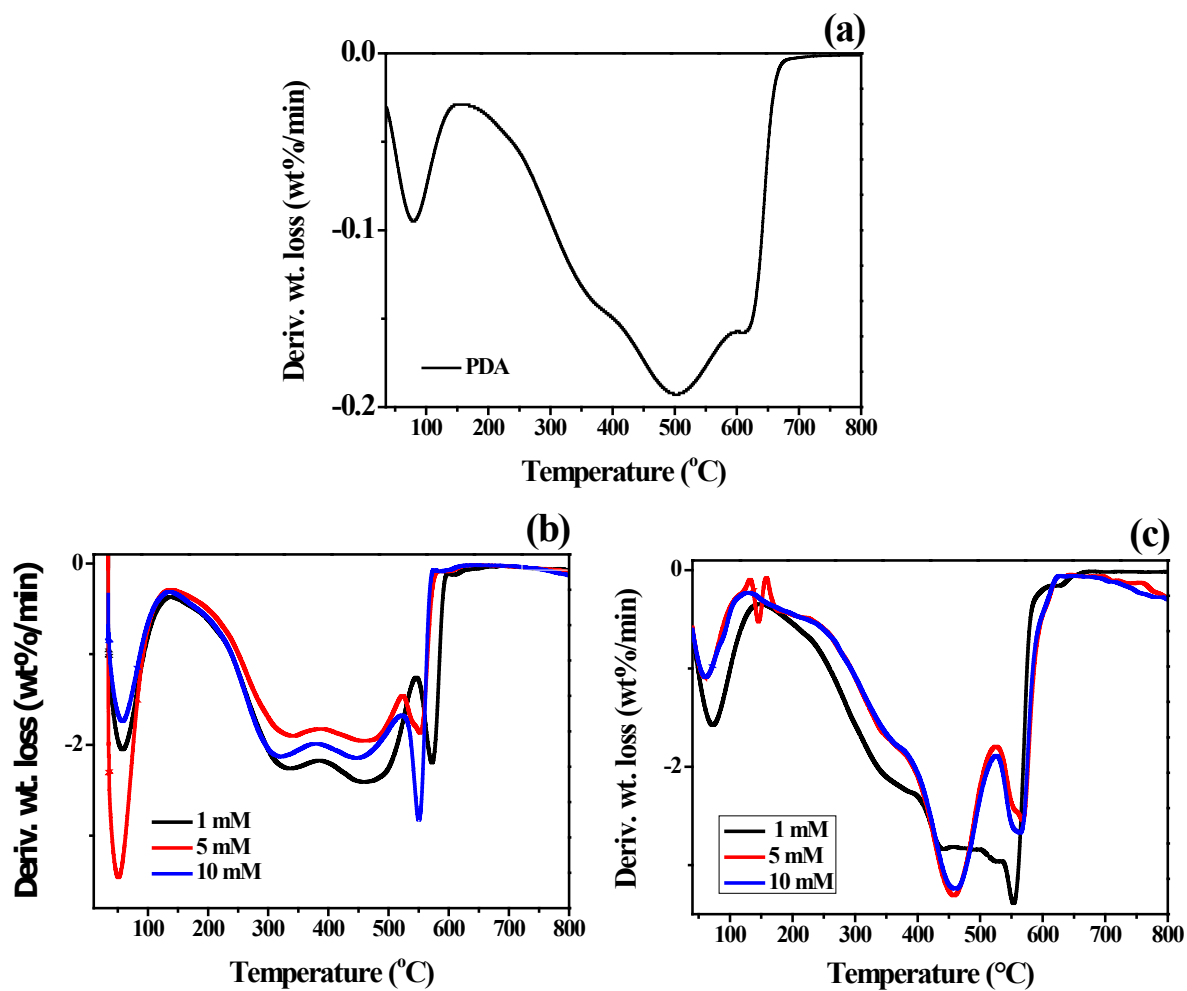


Figure S4.5: DTGA of (a) PDA. (b) Ag-PDA and (c) Ag@PDA nanocomposites at different AgNO₃ concentrations.

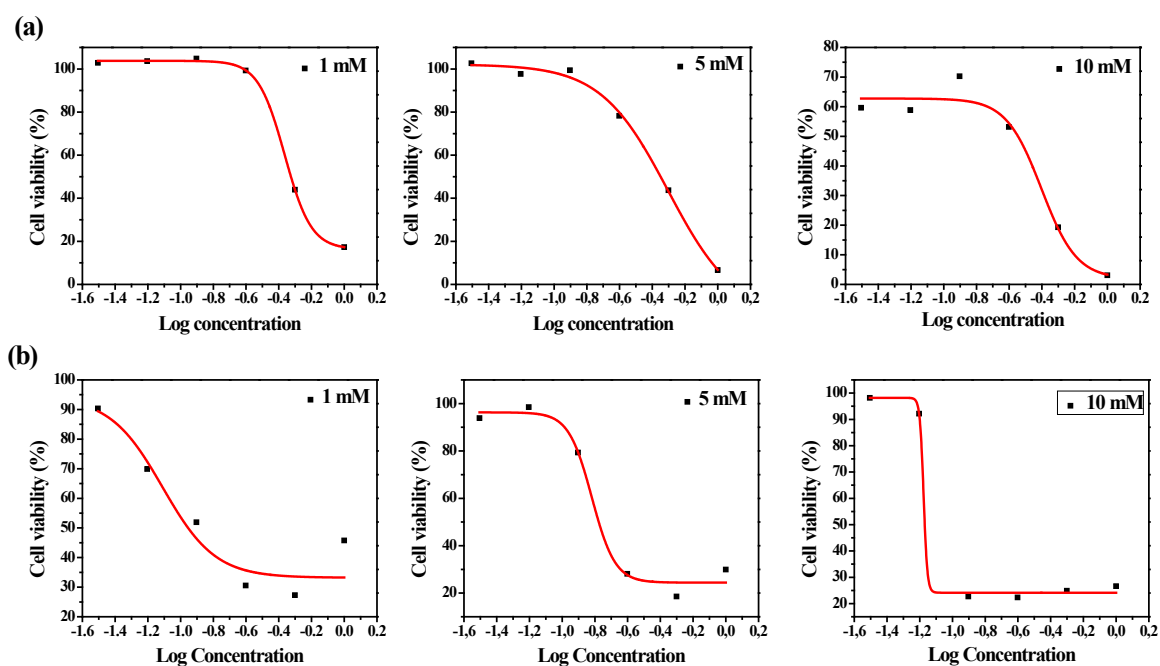


Figure S4.6: Sigmoidal curves used to determine the CC_{50} values of (A) Ag-PDA and (B) Ag@PDA against BHK-21 cells at different concentrations of AgNO_3 .

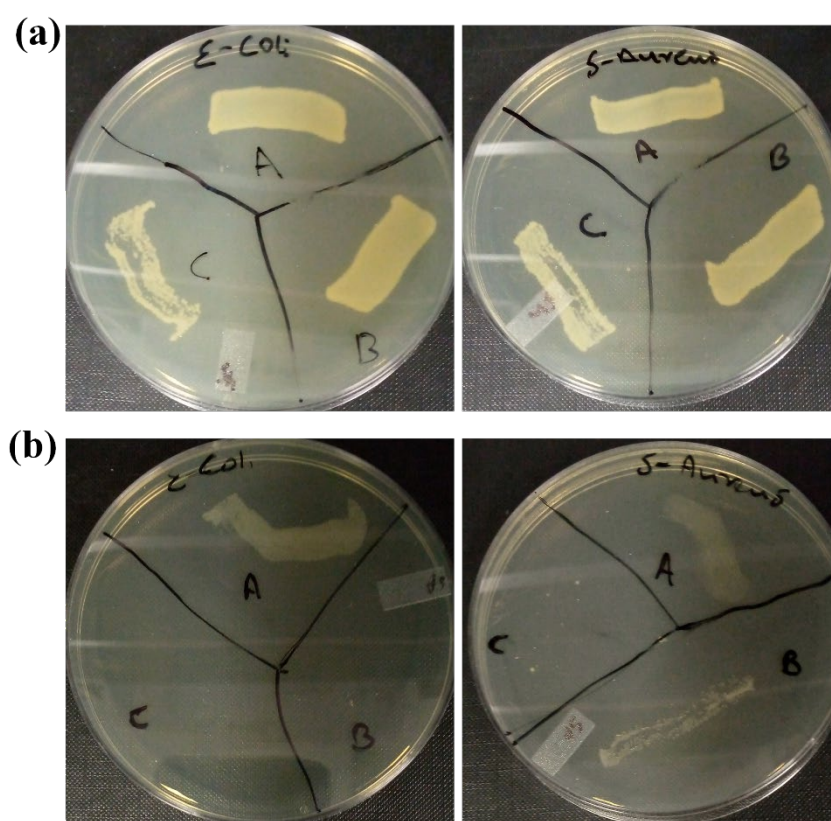


Figure S4.7: The MBC of (a) Ag-PDA and (b) Ag@PDA nanocomposites at different AgNO_3 concentrations [(A) 1 mM, (B) 5 mM and (C) 10 mM].

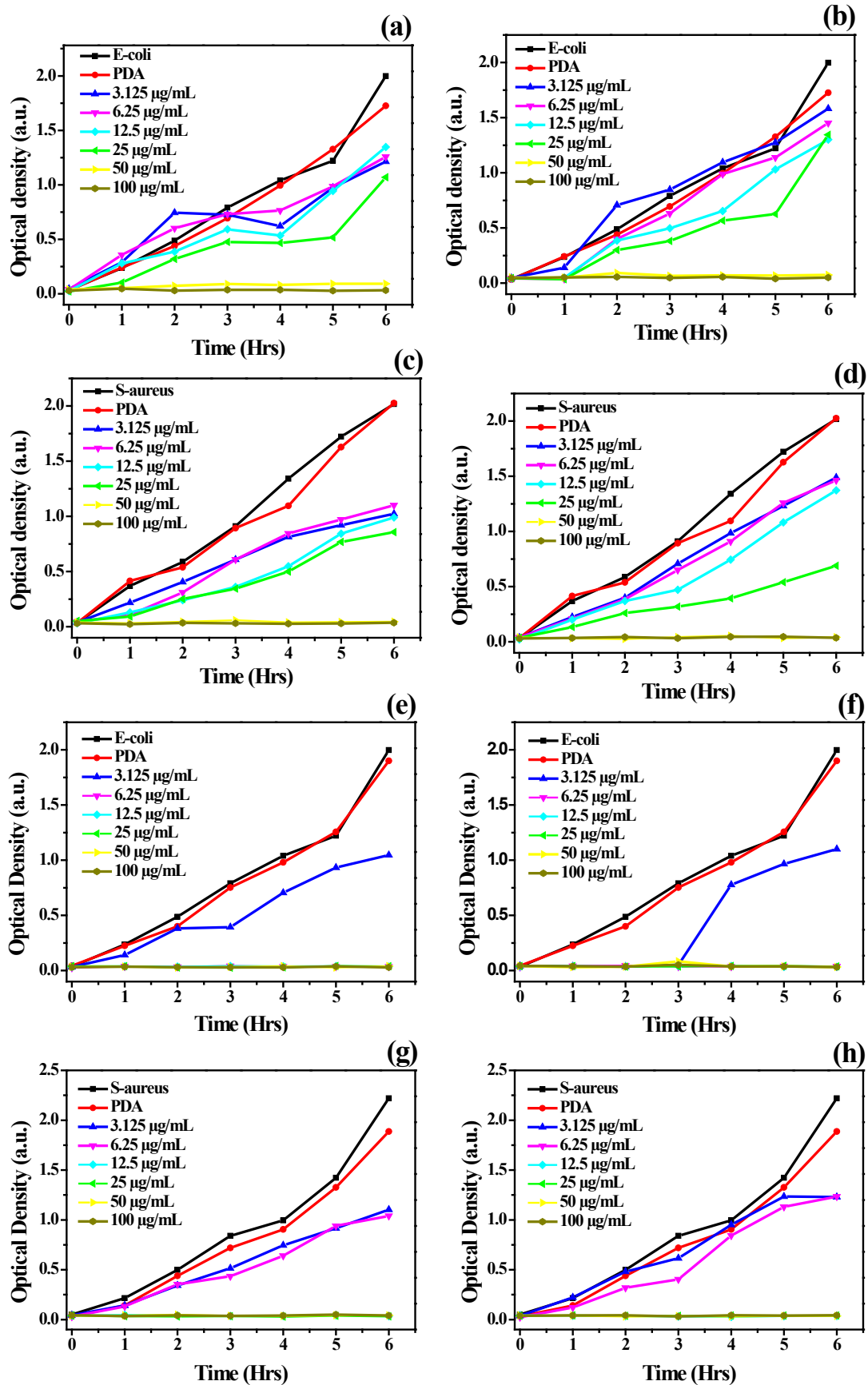


Figure S4.8: The bacterial growth curves of (a-d) Ag-PDA and (e-h) Ag@PDA against *E. coli* and *S. aureus*.

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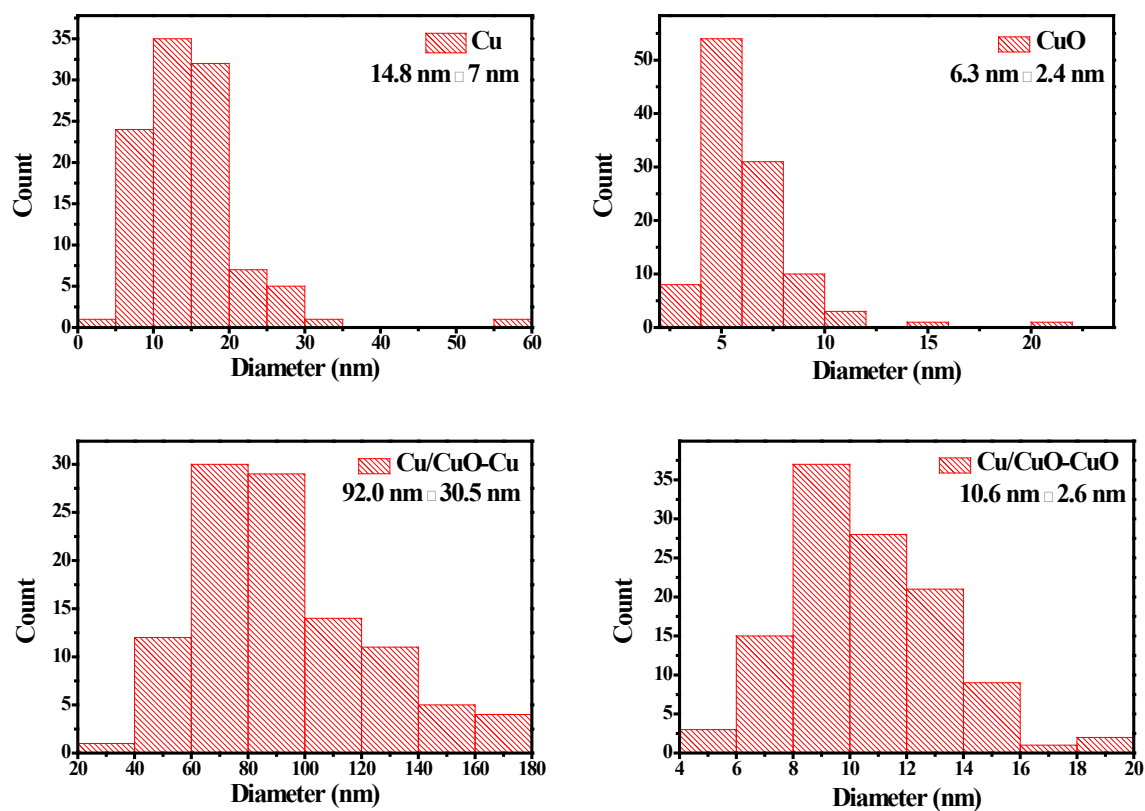


Figure S5.9: Size distribution histograms of Cu, Cu₂O and Cu/Cu₂O NPs.

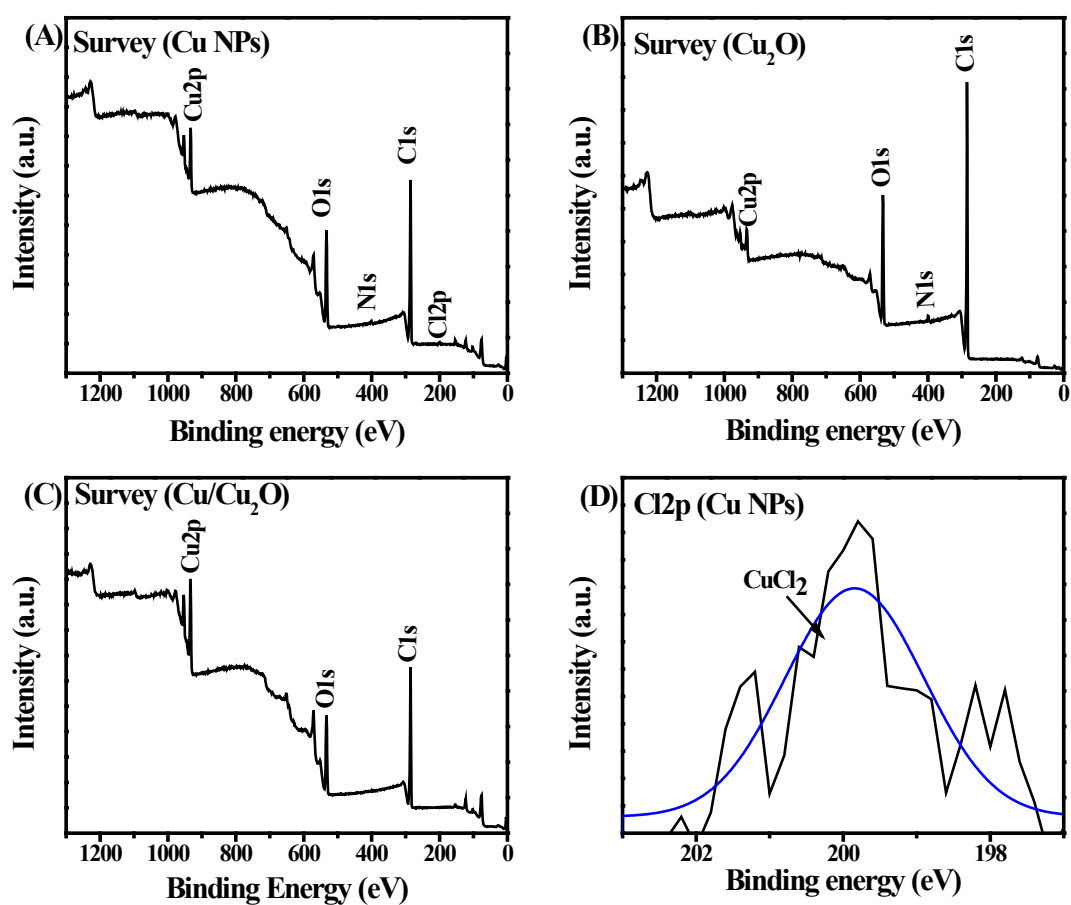


Figure S5.10: The XPS survey spectra of (A) Cu NPs, (B) Cu₂O NPs and Cu/Cu₂O NPs together with (D) Cl 2p spectrum for Cu NPs.

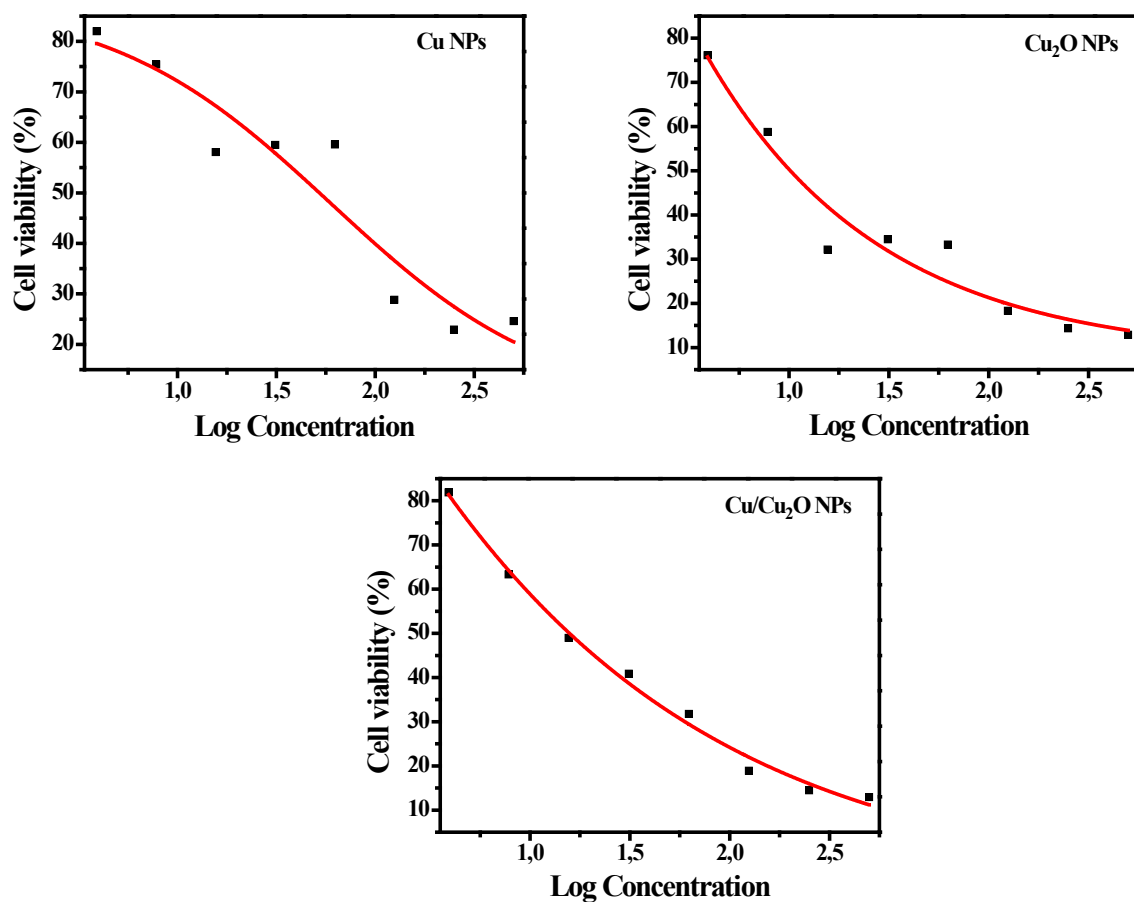


Figure S5.11: The sigmoid curves of cell viability acquired by incubating BHK-21 cells with (A) Cu NPs, (B) Cu₂O and (C) Cu/Cu₂O NPs at different concentrations.

Chapter 7: Supporting information

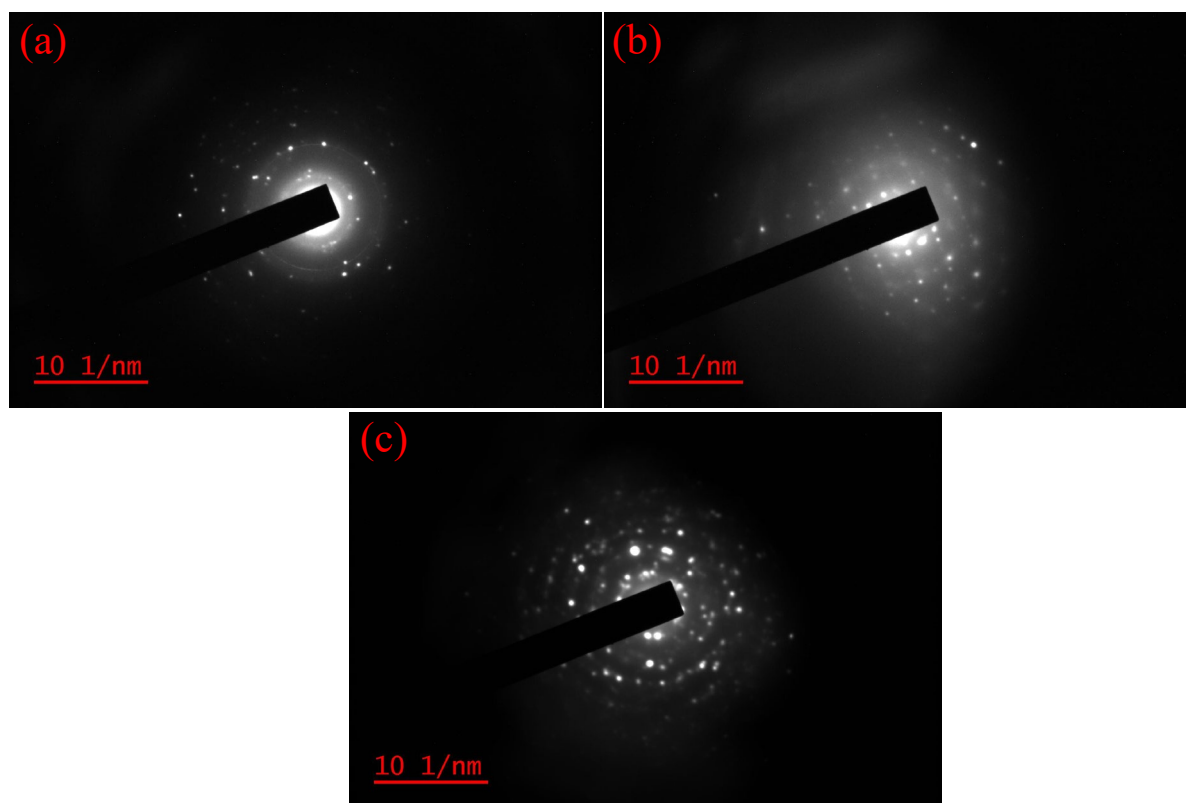


Figure S7.1: SAED images of (a) pristine Cu, (b) pristine Ag and (c) BNF-1.